NATIONAL INSTITUTE OF HEALTH

JOURNAL OF THE NATIONAL CANCER INSTITUTE

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VOLUME 4 *June 1944* NUMBER 6

FEDERAL SECURITY AGENCY
UNITED STATES PUBLIC HEALTH SERVICE
WASHINGTON, D. C.

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Published by the Federal Security Agency in accordance with section 220, title 44, U. S. Code.

Approved by the Director, Bureau of the Budget, as required by Rule 42 of the Joint Committee on Printing.

UNITED STATES GOVERNMENT PRINTING OFFICE, WASHINGTON : 1944

For sale by the Superintendent of Documents, U. S. Government Printing Office, Washington 25, D. C. - Price 40 cents a copy

Subscription price per year in the United States, Canada, and Mexico, $2

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NATIONAL CANCER INSTITUTE
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PRODUCTION OF MALIGNANCY IN VITRO. VIII. OBSERVATIONS ON THE MITOCHONDRIA AND GOLGI MATERIAL

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INTRODUCTION

Previous papers in this series (*1*, *2*, *3*) dealt with the isolation of fibroblasts in tissue culture, their reaction to treatment with methylcholanthrene, and a description of the tumors produced following the inoculation of mice with the treated cells. The primary aim of the present study was to examine the mitochondria and Golgi material of these fibroblasts exposed to methylcholanthrene. In addition, it was felt that a secondary aim might well be to obtain further evidence as to the frequency of the occurrence of typical Golgi material in cells grown in vitro under what may be considered optimum conditions, according to present-day standards.

Cytologists today would not question the reality of mitochondria, nor would they question the presence of mitochondria in cells grown in vitro. However, rather consistently negative results were obtained by early investigators in their attempts to demonstrate the presence of Golgi material in cells grown in vitro. This was considered by some (*4–6*) as support for the view that the Golgi material, invisible in living somatic cells both in vivo and in vitro, is a fixation artifact. One of the implications of this view is that the Golgi material has no real counterpart in the living cell and that hence it can be of no importance in cell physiology.

The results of Richardson (*7*), Macdougald and Gatenby (*8*), Hill (*9*), and Macdougald (*10*, *11*) showed that by the use of classical methods Golgi material may be demonstrated in fibroblasts and other cell types in vitro. Analysis of the results of these investigators indicated that the Golgi material became more readily demonstrated in vitro as the technique of tissue culture improved. Richardson noted that fragmentation of the Golgi material preceded the appearance of other cytoplasmic changes indicative of a pathologic state. This fact suggested that, far from being a mere fixation artifact, the Golgi substance is a sensitive indicator of cell damage.

MATERIAL AND METHODS

It was found necessary to use lying-drop cover-slip preparations in the studies in vitro since the osmiophilic and chromophilic character of the plasma clot precluded the use of the flask cultures described previously (*1*). These slide cultures were planted from flask cultures of about 30 days' growth. Small strips (1×7 mm.) were cut from the flask cultures, and one strip was planted in one drop of chicken plasma and two drops of a 40-percent horse serum, 20-percent embryo juice, and 40-percent saline solution spread over the surface of a No. 1 cover slip, 22 mm. square. The cover slip was attached by a small drop of culture fluid to a cover slip 48 mm. square and 0.4 mm. in thickness, which in turn was cov-

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ered by a hollow ground Pyrex slide having a cavity 40 mm. in diameter and 3 mm. deep. The large cover slip was attached to the slide by means of a thick ring of heavy yellow vaseline (melted over a water bath), while an outer seal of paraffin served to secure the preparation further. The preparations were fixed after approximately 64 hours' incubation at 38° C.

Preparations were obtained from flask cultures exposed to methylcholanthrene for various intervals (table 1). Tumors derived from the injection of tissue-culture clumps were also studied. The samples used were taken from tumors resulting from the intramuscular injection of cultures of cell strains D, H, J, L, N, and O, which had been exposed to the carcinogen 0 (control), 6, 32, 111, 184, and 406 days, respectively. These tumors were fixed on December 5, 1942, and February 1, 1943.

TABLE 1.—*Strains from which slide cultures were prepared*

Strain	Date fixed	Time in carcinogen	Time in vitro	Time since removal from carcinogen
	1941	*Days*	*Days*	*Days*
208 experimental	Dec. 7	123	415	0
208 control	Dec. 7	0	415	0
202D experimental	Dec. 21	16	429	0
202D control	Dec. 21	0	429	0
202E experimental	Dec. 29	10	437	0
202E control	Dec. 29	0	437	0
	1942			
202F experimental	Jan. 9	28	448	0
202F control	Jan. 9	0	448	0
202G experimental	Jan. 11	30	450	0
202G control	Jan. 11	0	450	0
202D experimental	Jan. 25	51	464	0
202D control	Jan. 25	0	464	0
202G experimental	Mar. 16	94	514	0
202G control	Mar. 16	0	514	0
208 experimental	Mar. 23	229	521	0
208 control	Mar. 23	0	521	0
	1943			
202H	May 14	6	938	519
202J	May 14	32	938	493
202L	May 14	111	938	409
208N	May 14	184	938	462
208O	May 14	406	938	240
202D	May 14	0	938	0
220A, Nos. 504, 506	Nov. 22	0	30	0
220A, Nos. 604, 610	Nov. 22	0	30	0
221–1	Nov. 22	0	30	0
221–2	Nov. 22	0	30	0

The methods used for the demonstration of mitochondria and the Golgi material were described previously (*12*). However, in dealing with tissue cultures, certain modifications of the original methods were necessary. The usual Heidenhain iron-hematoxylin method for staining mitochondria could not be used on the original tissue-culture clots because of the highly chromophilic nature of the plasma. Even in areas where the clot was fairly thin, little success was obtained since the cells at the very periphery of the growth zone alone could be differentiated. To obtain consistently satisfactory results, the following procedure was carried out routinely on all tissue-culture preparations reserved for the study of mitochondria: The cover-slip cultures were fixed in Regaud's fluid warmed to 38° C. This first fluid was changed at the end of one-half hour and fresh Regaud's fluid added, after which the cultures were placed in a cold room at 3° to 5°. Fixation was carried out for 3 days at this temperature, the fluid being changed every 24 hours. At the end of the third day of fixation, the cultures were rinsed in distilled water and transferred to 5 percent potassium dichromate and placed in an incubator at 38° for 3 days. During the period of chromation, the cover-slip dishes were sealed with petroleum jelly; afterwards the cultures were washed in tap water for 1 hour. After washing, the cover slips were placed on microscope slides, and the original explant and the excess plasma were dissected away. Thus, only the cells which had grown out from the original explant and which had become attached to the cover slip were retained. These cells retained after dissection were arranged in a more or less single layer, an arrangement that allowed for satisfactory staining with hematoxylin and for differentiation with iron alum. The cultures were placed in 4 percent ferric ammonium alum at room temperature for approximately 16 hours, rinsed quickly in distilled water, and then transferred to 1 percent aqueous hematoxylin, which was prepared by adding 1 gm. of hematoxylin to 100 cc. of distilled water. The mixture was brought to a boil, allowed

to simmer for 1 minute, and cooled to room temperature. The stain was then ready for use and when kept in a refrigerator remained usable for several weeks.[1] After staining, the cultures were washed in distilled water to remove the excess stain and were then transferred to 2 percent ferric ammonium alum for differentiation under the medium power of the microscope. The majority of the cultures were differentiated for mitochondria, but a few from each series were differentiated so as to bring out other cytoplasmic constituents.

The cultures used for demonstration of the Golgi material were fixed in Champy's fluid (1 part 1 percent chromic acid, 1 part 3 percent potassium dichromate, and 1 part 2 percent osmic acid) for 24 hours, washed in running tap water for 24 hours, and placed in 2 percent osmic acid and incubated at 38° C. for 5 days. The cover-slip dishes were sealed with petroleum jelly during the period of osmication. After osmication, the cultures were washed in running tap water for 1 hour. Dissection of the original transplant and of the excess plasma was carried out at this stage. This procedure was followed when it was found that the plasma and the Golgi material were bleached equally with potassium permanganate and oxalic acid.

It should be emphasized that all slide cultures were fixed immediately after removal from the incubator, without an intervening wash in saline solution. It had been found previously (*13*) that immersion of tissues in warm Locke's solution for very short intervals caused a rapid fragmentation of the Golgi material. While many of the fixatives commonly used in tissue-culture studies require such a preliminary rinse, the fixatives deliberately selected for the present studies do not produce any marked flocculation or precipitation in the clot. It was therefore possible to eliminate entirely the complications resulting from a saline rinse.

Fixation of the tumor tissue derived from the inoculation of tissue-culture clots was carried out as previously described (*12*). Mitochondria in these sections were stained with the Bensley-Cowdry stain as well as with Heidenhain's iron hematoxylin.

All photomicrographs were taken at a magnification of 1300 ×.

RESULTS

TISSUE-CULTURE PREPARATIONS

Mitochondria.—Control cultures and cultures exposed to the action of 20-methylcholanthrene for the various intervals indicated were compared in detail to determine whether any significant and consistent changes in mitochondrial form or number could be detected. Under certain conditions, fragmentation and fusion of mitochondria were noted, but in such cases the changes could be attributed equally to the presence of necrosis in the cell clump or to other adverse environmental conditions. Figure 1, *A*, shows the pattern of the mitochondria characteristic of the growth zone in normal[2] control cultures. The mitochondria are fairly large, moderate in number, filamentous, and tend to be oriented along the long axes of the cell processes. Figure 1, *B*, shows the mitochondria present in cells flattened against the cover slip near the center of a control culture. Because of the thin film of cytoplasm in such cells, the mitochondria were more readily differentiated.

When compared with the controls, the cells of cultures exposed to methylcholanthrene for the shorter intervals showed no consistent differences in mitochondrial pattern. However, after longer exposure many cells showed certain differences from the controls. Figure 2, *A*, shows a cell from a culture

[1] This method of artificially ripening hematoxylin has given consistently good results in our hands and has the advantages of being easily prepared and of never being overripened or underripened. In addition, staining and differentiation times are easily standardized.

[2] These cultures were considered normal since they were fixed long before the demonstration of malignancy on inoculation into normal mice and before there was any evidence of a change in growth characteristics in flask cultures (*1*).

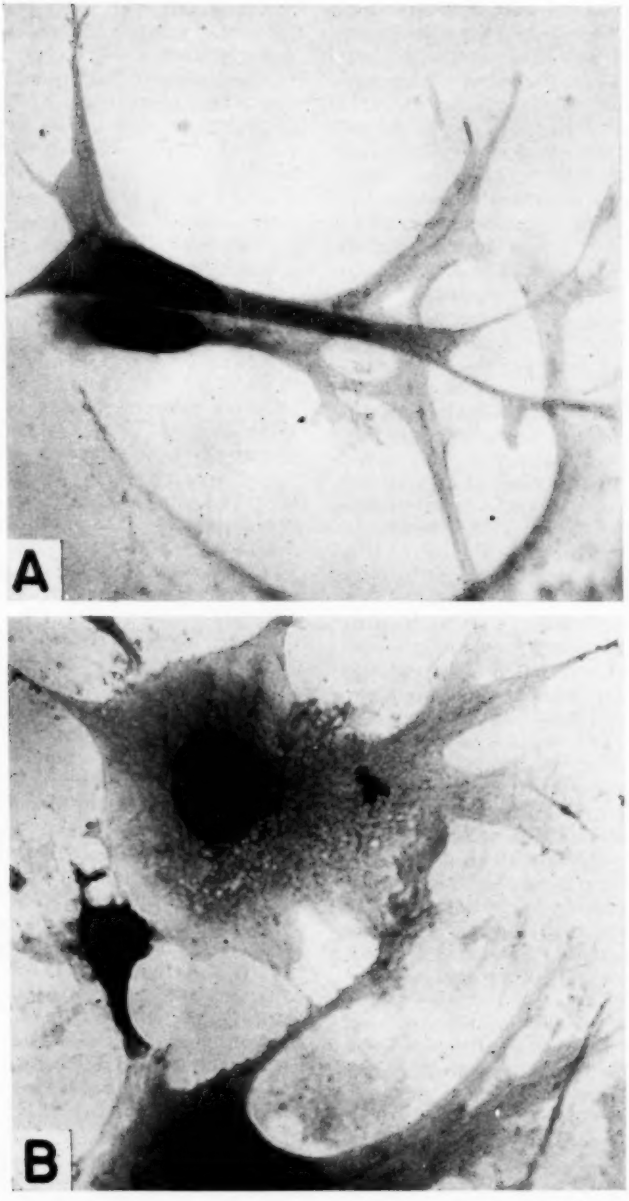

FIGURE 1.—Mitochondria in cells of a culture from control series 202E. *A*, Cells in the growth zone: *B*, Cells near the center of the culture. Regaud fixation and Heidenhain's iron-hematoxylin stain.

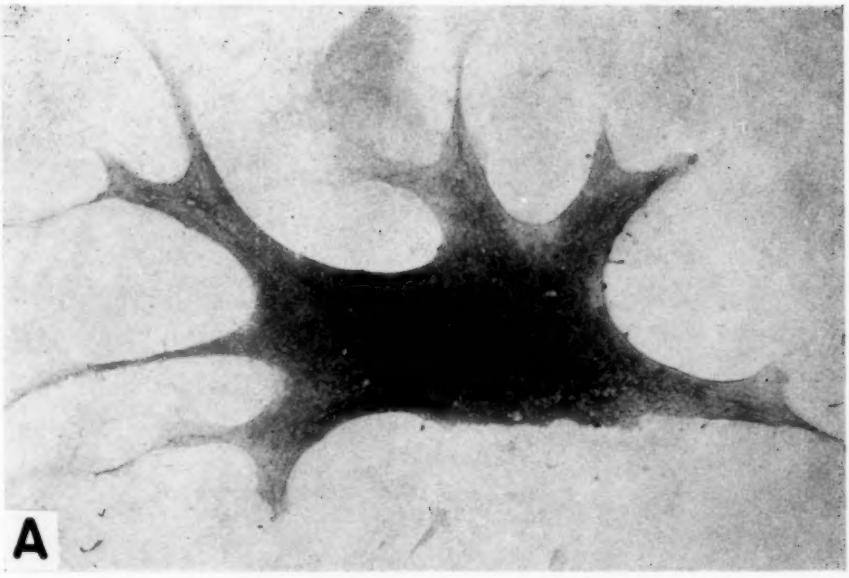

FIGURE 2.—*A*, Mitochondria in a cell near the center of a culture from series 2020 exposed to methylcholanthrene 406 days; *B*, Cells containing mitochondria and numerous, small, chromophilic droplets equal in size, in a culture from the same series as *A*. Regaud fixation and Heidenhain's iron-hematoxylin stain.

exposed to the carcinogen for the longest interval (406 days). The mitochondrial pattern is typical of many of the cells in such cultures. The mitochondria still retain their filamentous form but tend to be clumped, and they appear to be somewhat smaller. This clumping interferes but slightly with the typical orientation along the long axes of the cell processes. The granulations in the large central area of the cell are not to be confused with the filamentous mitochondria visible mainly at the periphery.

The formation of numerous small droplets, chromophilic to hematoxylin, while occurring in control cultures, is much more common in cells of cultures exposed to methylcholanthrene for long intervals (fig. 2, *B*). In addition to the more frequent occurrence of granules in cells of strain O, the cytoplasm appeared to be more dense and to require more time for differentiation after staining in iron hematoxylin than that of cells of other series.

In both control cultures and those exposed to the carcinogen, cells stained to demonstrate mitochondria frequently contained long intracellular fibrils (fig. 3), which occasionally appeared to extend beyond the cytoplasmic margins. These fibrils have been tentatively identified as fibroglia.

Golgi material.—The morphology of the Golgi material in cells of control cultures and in those exposed to the carcinogen was found to be essentially similar. However, differences in the type of Golgi network were found regularly within individual cultures. These differences appeared to be related to the position of the cells within the clump. Figure 4, *A*, shows the position and arrangement of the Golgi material in a cell at the margin of the growth zone in a control culture. It is in close contact with the nuclear membrane, and the network appears so compact that the indiyidual strands cannot be identified. Figure 4, *B*, shows the open network characteristic of the Golgi material in cells flattened against the cover slip near the center of the cell clump in a control culture. Parts of the network are in close contact with

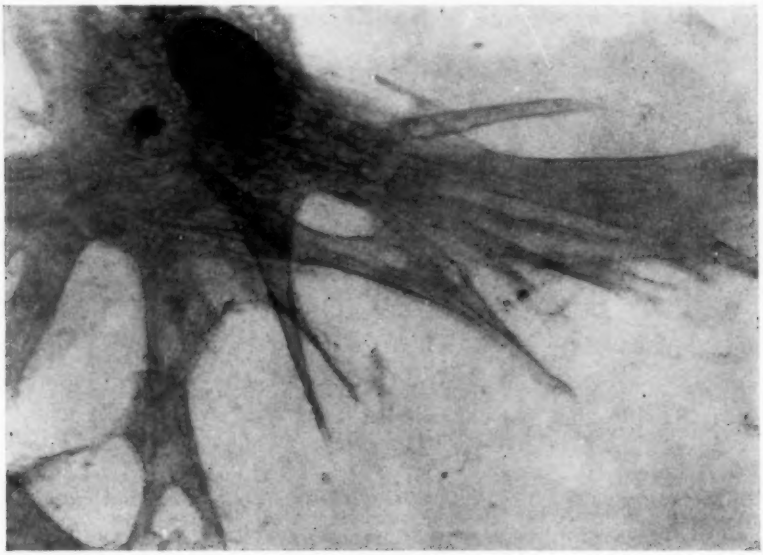

FIGURE 3.—Fibroglia fibrils in a cell of a culture from control series 202E. Regaud fixation and Heidenhain's iron-hematoxylin stain.

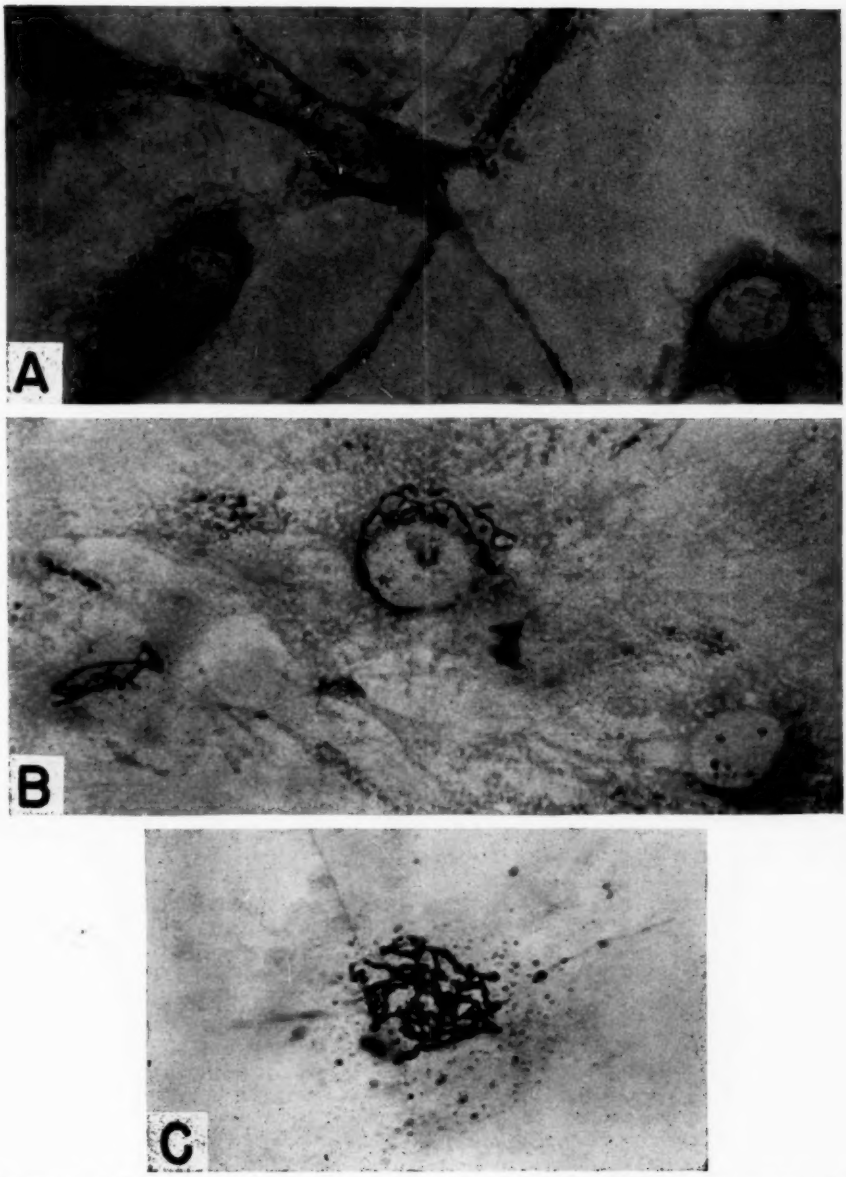

FIGURE 4.—Golgi material. *A*, In cells of the growth zone of a culture from control series 202E; *B*, In a cell near the center of a culture from the same series as *A*; *C*, An example of overimpregnation in a cell near the center of a culture from control series 202G. Champy fixation and osmic acid impregnation.

the nuclear membrane, but other portions extend for some distance into the cytoplasm. Figure 4, *C*, demonstrates an instance of overimpregnation of the Golgi material, in this particular case in a cell in a location similar to that shown in figure 4, *B*, from another control preparation. It is obvious that with overimpregnation the cytoplasmic particles that are not part of the Golgi complex become impregnated. The small spaces surrounded by osmiophilic material may appear to resemble the Golgi "internum" of Hirsch (*14*), but careful inspection of all cultures in the present series suggests that a true internum in the sense of Hirsch is not present. Figure 5, *A*, from a culture exposed 17 days and figure 5, *B*, from a culture exposed 229 days demonstrate the normal pattern of the Golgi material in cells exposed to the carcinogen for varying lengths of time. No essential differences in the pattern of the Golgi material exist between that of control and exposed cells.

Abnormalities in cell structure and in the pattern of the Golgi material were observed, particularly in the cultures exposed to methylcholanthrene. Degenerative changes in the Golgi material were noted in occasional cells in both control and exposed cultures, however. Figure 6, *A*, shows the location of apparently normal Golgi material in a binucleate cell from a culture exposed 229 days. Figure 6, *B*, demonstrates the bipolar arrangement of the Golgi substance which, while rare, was found in both control and exposed cultures. Figure 7, *A*, *B*, and *C*, shows different stages in the fragmentation and dissolution of the Golgi material. Figure 7, *A*, from a culture exposed 229 days, shows the beginning of fragmentation in which the network, still partly filamentous, appears to be breaking up into granules of various sizes. Figure 7, *B*, shows a binucleate cell in a culture exposed 17 days. The Golgi substance in this instance was preserved in the form of granules of various sizes. Figure 7, *C*, shows the ring-shaped form of the Golgi material which is frequently found in cells located near areas of necrosis. This particular example was taken from a culture exposed 17 days. These examples of fragmentation and dissolution of the Golgi material were all taken from cultures exposed to the carcinogen, but similar examples could have been chosen from control cultures. The impression was gained, however, that while present in control cultures, fragmentation was more frequently encountered in cultures exposed to the carcinogen for short (17 days) or long (406 days) intervals. Control cultures and those exposed to the carcinogen for intermediate intervals, particularly 111 days, appeared to be relatively free of fragmentation.

Early in the present series of studies (*1*), it was noted that the rapid outward migration of individual cells, which give the characteristically indefinite and loose margin to the growth zone in control cultures, was interfered with in some way in cultures exposed to methylcholanthrene. In the latter series, especially during long-continued exposure, cultures exhibited a very sharp, easily defined margin. This was particularly true of that part of the culture growing near or in contact with the surface of the flask. These cultures gave the impression of an "epithelial-like" rather than of a "fibroblast-like" type of growth, with their closely packed cells, scant intercellular space, and clearly defined culture margins.

The location of the Golgi material is considered by some to give evidence of cell polarity, particularly in the case of epithelial cells. Hence, it was felt that some interesting information might be obtained by determining the location of the Golgi material in relation to the nucleus in a given number of cells of the growth zone in both control and exposed cultures. Counts were made on 1 control set of cultures and on sets exposed 6, 32, 111, and 406 days. These slide cultures were fixed May 14, 1943. In each case the Golgi material was listed as being distal, i. e., on the side of the nucleus away from the center of the clot; lateral; or proximal, i. e.,

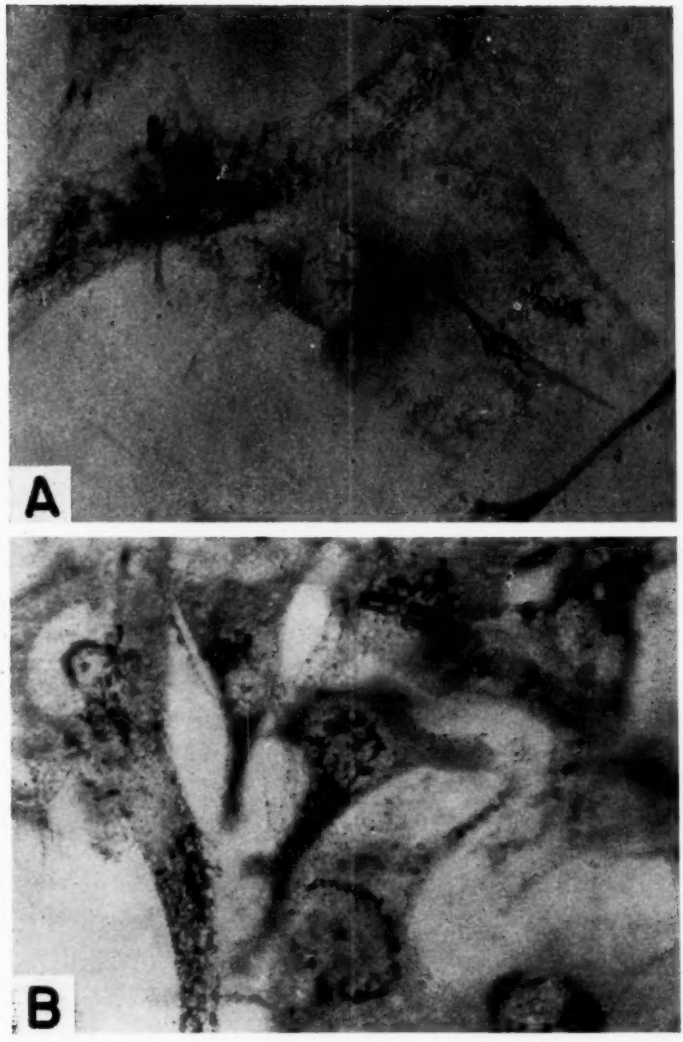

FIGURE 5.—Golgi material. *A*, In cells near the center of a culture from series 202D exposed to methylcholanthrene 17 days; *B*, In cells near the center of a culture from series 208 exposed to the carcinogen 229 days. Champy fixation and osmic acid impregnation.

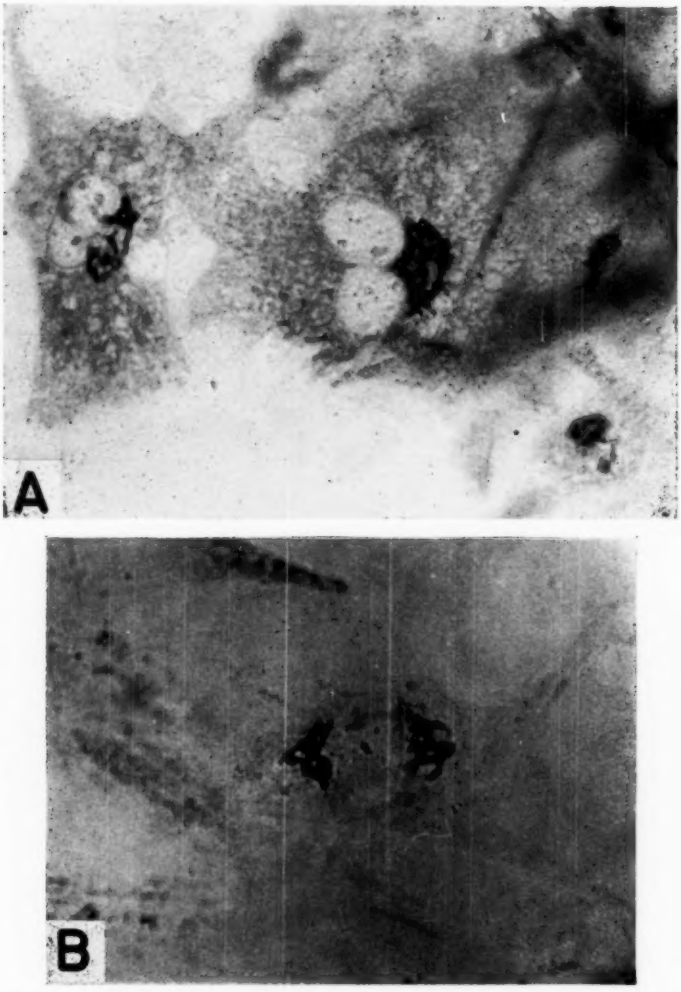

FIGURE 6.—*A*, Orientation of the Golgi material in a binucleate cell of a culture from series 208 exposed to methylcholanthrene 229 days; *B*, Bipolar Golgi material in a cell of a culture from control series 202E. Champy fixation and osmic acid impregnation.

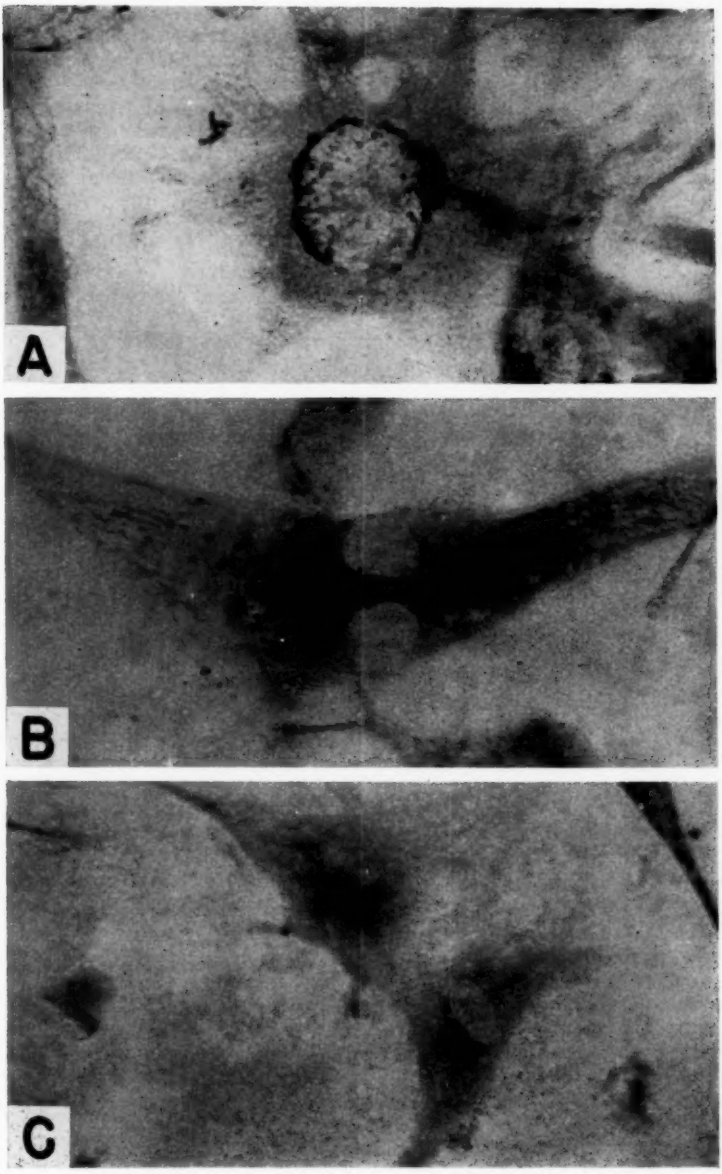

FIGURE 7.—Fragmentation and disappearance of the Golgi material. *A*, Beginning fragmentation in a cell near the center of a culture from series 208 exposed to methylcholanthrene 229 days; *B*, Later stage showing its granular character in a binucleate cell from series 202D exposed to the carcinogen 17 days; *C*, Small ring-shaped remainder of the Golgi material in a cell from the same series as *B*. Champy fixation and osmic acid impregnation.

at the pole of the nucleus near the center of the clot. The counts were made on 100 cells from each of 2 slides in the different series. The significant results are presented in figure 8. The control series contained the greatest number of cells possessing distally oriented Golgi material. Next in order was the series exposed to methylcholanthrene 111 days. The series exposed 406 days possessed the smallest number of cells with this orientation of the Golgi material.

In the material prepared for the demonstration of the Golgi substance it was noted that in both transplants of spontaneous tumors and those produced by inoculation of tissue-culture clumps there was a tendency towards hypertrophy of the Golgi substance. When compared with normal fibroblasts, the Golgi material appeared to extend into a larger area of the cytoplasm in the tumor cells. It was noted also that the Golgi material of the cells of the endothelium of the host blood vessels sup-

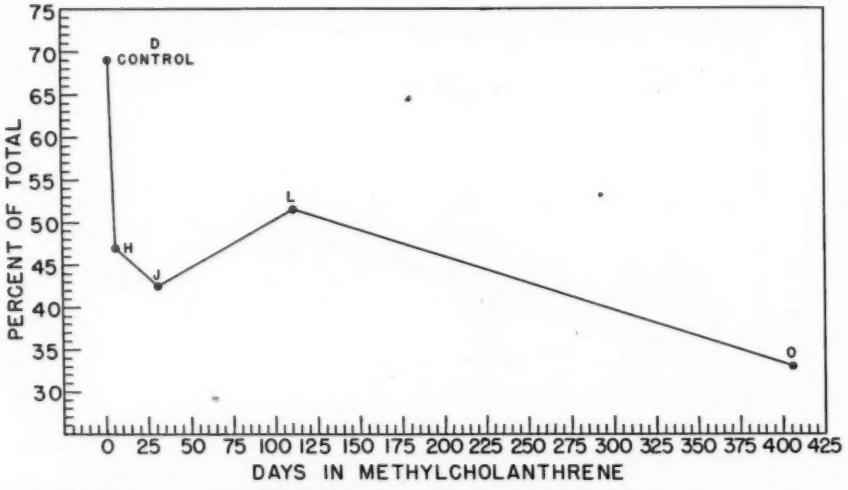

FIGURE 8.—Percentage of cells exhibiting distally located Golgi material in control series D and in exposed series H, J, L, and O. Ordinate, percent of total number of cells counted; abscissa, days in methylcholanthrene.

Transplanted Tumors.—Tumors derived from the subcutaneous or intramuscular inoculation of tissue-culture clumps from strains H, J, L, N, and O and control series D were submitted to cytologic examination. Sections of the tumors were compared with material containing normal fibroblasts and with sections of transplants of spontaneous fibrosarcomas. In the cells of tumors derived from the inoculation of tissue-culture cells, the mitochondria were regularly filamentous and fairly numerous. A similar pattern was found in normal fibroblasts and in the cells of the fibrosarcomas studied.

plying the tumors showed some hypertrophy, thus confirming the earlier observation of Ludford (*15*). The cells of tumors derived from the injection of tissue-culture clumps exposed to methylcholanthrene 406 days possessed rather distinctive cytologic characteristics. They uniformly contained a more dense cytoplasm, especially in the region of the Golgi material, than did the cells of any other tumors studied. In addition, many of the cells contained numerous, small, osmiophilic droplets of uniform size, which resembled those seen in the cells of the tissue-culture series exposed to the carcinogen 406 days.

Attempts were made to determine the orientation of the Golgi material in relation to cell groups within the tumors, but no definite evidence of such orientation could be found.

DISCUSSION

It would have been more satisfactory if flask cultures comparable with those used in previous studies (*1*) could have been used in the present investigation. The technical difficulties inherent in cytologic studies limited us to the use of slide cultures and to the cells located at the cover slip-plasma clot interface in these cultures. It is fortunate that the cells of this layer were found to display the greatest changes in growth pattern following exposure to methylcholanthrene (*1*). In the interpretation of results in the present study, this limitation was borne in mind.

Previous studies did not result in the establishment of any consistent differences in mitochondrial pattern between normal fibroblasts and fibrosarcoma cells, although Lewis (*6*, *16*) claimed that sarcoma cells commonly contain smaller and more numerous mitochondria than do normal fibroblasts in tissue culture. For a review of this subject, see Cowdry (*17*). In the present work, no clear-cut and consistent changes in mitochondrial form and number were noted in the cells exposed to the action of methylcholanthrene in tissue culture. Further, the loss of orientation and the tendency towards clumping of the mitochondria of cells exposed for long intervals to the action of the carcinogen is probably best explained as a purely mechanical interference with the natural pattern, because of the presence of numerous, small, chromophilic droplets in the large majority of such cells. Regardless of the nature or significance of these changes, they would not appear to be involved in the mechanism of neoplasia or of malignancy. Malignancy, as judged by the percentage of tumors produced by the inoculation of tissue-culture clumps into mice (*2*), and the relative frequency of metastases (*2*, *3*), reaches its highest level in cells exposed for much shorter intervals than those required to produce the changes just discussed.

Insofar as the attempt to correlate neoplastic and malignant change with cytologic changes is concerned, the study of the Golgi material produced results more completely negative than did the study of the mitochondria. This is perhaps support for the view of Ludford (*18*, *p. 256*):

> The structure of the Golgi apparatus of malignant cells corresponds to that of their prototypes or to stages in their differentiation.

The fact that no differences have been noted in the pattern of the Golgi material in normal and malignant fibroblasts in tissue culture is not surprising. Striking differences in the distribution of the Golgi material have been noted between normal hepatic cells and hepatoma cells (*12*); in this case, partially peripheral Golgi material in the normal cell becomes juxtanuclear in the hepatoma. However, in fibroblasts Golgi material is normally juxtanuclear.

The clear correlation between the rate of increase in width of the cultures and the number of cells exhibiting a distal polarization of the Golgi material is interesting. The graph demonstrates marked similarity to curves for the rate of increase in width of similar cultures in vitro (*1*), for the percentage of tumors arising from inoculation of these cultures into mice (*2*), and for the anaerobic glycolysis of slices of these tumors (*19*). It is conceivable that cellular changes induced by the presence of a toxic substance, e. g., change in cellular orientation, might remain in evidence after removal of the substance from the culture medium. It seems more reasonable to assume that such changes are expressions of apparently permanent alterations induced in the cells by the carcinogen. All cultures in this series were originally derived from one strain of fibroblasts, and the one factor known to

have been varied was the length of exposure to methylcholanthrene. If these variations from the controls had resulted entirely from the nonspecific toxic action of methylcholanthrene (in contradistinction to its specific carcinogenic action), a reassertion of the characteristics of control cultures and cells might have been expected following the removal of the cells from the carcinogen. Such a reversion did not take place, and therefore there seems little reason to assume that these changes were induced by a toxic action of methylcholanthrene.

The fact that a correlation between the rate of increase in width of the culture, or migration rate of the cells, and the number of cells exhibiting a distal polarization of the Golgi material is interesting in itself. It suggests that while fibroblasts do not possess the permanent polarity of epithelial cells in vivo, they nevertheless possess a temporary but definite polarity related to the direction of movement, the position of the Golgi material being an indicator of the direction of cell migration at the time of fixation.

Evidence is presented which supports the view of those who consider that the Golgi material is an essential cell structure, and that it has as much reality as the nucleus or the mitochondria. In the first place, the ease with which the Golgi material has been demonstrated in all the cultures studied is reason enough for removing tissue-culture cells from the small group of vertebrate cells in which Golgi material has not been consistently demonstrated. Consistent differences in the pattern of the Golgi material contained in the cells of the growth zone and in the cells flattened against the cover slip in the center of the culture is added evidence of the reality of the Golgi substance. It is hard to believe that artifacts so consistently different in character could be produced regularly in the same regions in a large series of slides. The correlation between the rate of migration and the number of cells containing distally oriented Golgi material in tissue culture indicates an association between cell polarity and the Golgi material. This is further evidence in support of the view that the Golgi material, an area of highly reactive cytoplasm, which is visualized in the process of replacement by heavy metals, is a very real part of the vertebrate cell.

A comparison of the techniques employed and results obtained by earlier investigators (*7–11*) with those presented herein indicate that the successful impregnation of the Golgi material of cells in tissue culture is more closely correlated with the tissue-culture methods employed than it is with the methods used for demonstrating the Golgi substance. However, there is a further factor that probably enters into the technical difficulties encountered in demonstrating Golgi material in tissue-culture cells. Fixatives (such as Champy's fluid) which contain mixtures of osmic acid and potassium dichromate almost invariably fail to preserve properly the outer 50μ to 100μ of any tissue fixed in them. This fact is of little consequence when normal tissues and organs are being studied but obviously adds to the difficulties when tissue-culture clumps are being investigated. Such material is frequently not more than 300μ in thickness and in the zone of growth may be but one or two cells thick.

The use of plasma clots averaging ½ to 1 mm. in thickness in the present series was no doubt partly responsible for the relative ease with which satisfactory preparations demonstrating typical Golgi material were obtained. Good examples of Golgi material were obtained from all areas of the clumps, and the most satisfactory ones were found in cells flattened out on the cover glass. Many of these cells were extremely thin, and yet there was little evidence of fragmentation of the Golgi substance in such cells. It would thus appear that the extreme thinning and flattening of cells at the cover glass-plasma clot interface is not an important factor in the fragmentation of

Golgi material as suggested by Ludford (*20*).

In none of the material studied could the "internum" of Hirsch (*14*) be identified. This substance is considered to be a relatively osmiophobic material synthesized or formed from the osmiophilic "presubstance" (*14*; *21, p. 118*) of the Golgi region. Macdougald (*11*) described it in fibroblasts in tissue culture, but careful inspection of the present material gave no evidence of it. Excellent opportunities for studying the structure of the Golgi material were found in the extremely thin cells at the cover glass-plasma clot interface in these preparations. Thin osmiophilic strands of the Golgi material frequently surrounded small areas of cytoplasm; but these areas did not appear to be in any way different from areas of cytoplasm outside the Golgi region, except that they were usually devoid of mitochondria (fig. 4, *B*). Apparently these cells should be classed with that group of cells that contain only the osmiophilic "presubstance" portion of the Golgi material.

Further evidence is presented confirming the results of Ludford (*20*) and Macdougald (*11*), who described the fragmentation of the Golgi material in damaged or dying cells in tissue culture. On the other hand, the present material contained no evidence favoring the suggestion of Ludford that the fragmented Golgi substance is the precursor of the fat of fatty degeneration in cells grown in tissue culture. Rather, it supports the view of Macdougald that there is no relation between the fragmentation of the Golgi substance and fat formation.

SUMMARY

Detailed descriptions are given of the methods used for the demonstration of mitochondria and the Golgi material of cells in tissue culture. Possible explanations for the failure of many previous investigators to demonstrate Golgi material in cells grown in tissue culture are also presented. It is felt that the tissue-culture methods employed are of considerable importance in this respect.

The mitochondria of all healthy tissue-culture cells, both in control cultures and in those exposed to methylcholanthrene, were found to be filamentous. The mitochondria of cells of tumors derived from the inoculation of tissue-culture clumps were also found to be filamentous.

No changes in mitochondrial pattern were noted following the exposure of tissue cultures to the carcinogen for the shorter intervals (6 to 111 days). The longest exposure, 406 days, resulted in many instances in a loss of orientation of the mitochondria. This change may have been related to the accompanying increased density of the cytoplasm and to the formation of numerous small chromophilic droplets in the cytoplasm.

In tumors derived from the inoculation of tissue-culture clumps, hypertrophy of the Golgi material was noted in the tumor cells as well as in the endothelial cells of the host blood vessels supplying the tumor. However, no differences in the pattern of the Golgi material were noted when cells of control cultures and those of cultures exposed to methylcholanthrene were compared.

The change in growth pattern of cells grown in tissue culture and exposed to methylcholanthrene was found to be correlated with the orientation of the Golgi material of cells in the zone of growth.

The presence of the Golgi "internum" of Hirsch in the Golgi material of fibroblasts could not be confirmed.

The presence of typical Golgi material in the cells of all cultures examined in the present study adds support to the view that this substance is present in some form in all healthy animal cells and that cells grown in tissue culture are no exception to this rule. Evidence of the manner in which the Golgi material disappears from the cell is presented. There was no indication that the disintegration of the Golgi material resulted in the formation of fat.

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CILIATED CELLS OF THE THYROID OF THE MOUSE

By THELMA B. DUNN, *research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service*

The observation of ciliated cells in the thyroid of the mouse was an incidental part of two experiments in which single sections of the thyroid from a large group of mice were examined. The experiments were not designed to show any effect on the thyroid; and the ciliated cells were obviously of natural occurrence since they appeared about equally in control and experimental animals. The thyroid attached to the trachea was removed immediately after death and fixed in Zenker-formol, followed by paraffin embedding, and routine hematoxylin-and-eosin staining. The cells were found in 10 of 22 strain C3H mice averaging about 10 months of age, and in 5 of 25 strain A animals of a similar age. Because of the frequent use of the mouse in cancer research, and because no previous report of such cells in the thyroid of the mouse has been found, a brief account of this observation seems warranted.

APPEARANCE OF CELLS

Andrew and Andrew (*1*) have described in detail the "senile involution" of the thyroid gland. In this process, large cystic follicles develop that contain a poorly stained substance in place of the usual colloid. Smith and Starkey (*2*) have likewise described age changes in the mouse thyroid, in which large areas of cystic degeneration appeared in the center of the gland where only remnants of the original secretion remained. None of these authors mention ciliated cells, but it was in just such follicles as these that they were most readily found. They have not been seen in follicles containing a homogeneous, acidophilic colloid throughout, although they are occasionally found where only a minor degree of regressive change, with vacuolization of the colloid, has occurred. They were seen clearly with a high magnification (450×) and had to be looked for carefully. They appeared singly or in short rows scattered among the often flattened cells lining the follicles that showed senile involution (figs. 1 and 2).

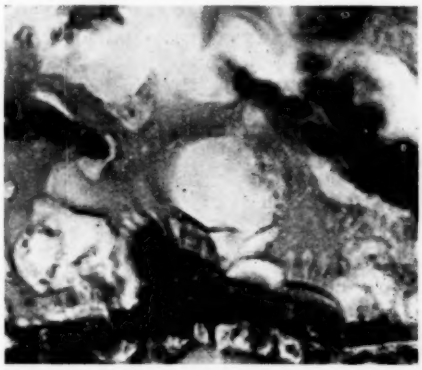

FIGURE 1.—Two ciliated cells near bottom of field; the openings between follicles are shown. Hematoxylin and eosin stain. ×1370.

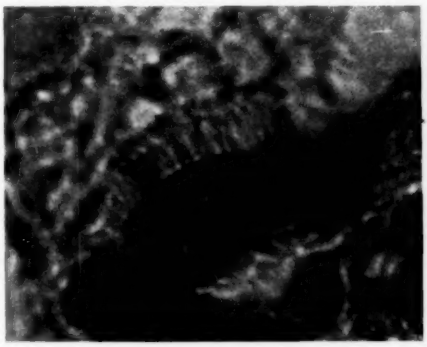

FIGURE 2.—Oil-immersion view of a single ciliated cell. Hematoxylin and eosin stain. ×3800.

The substance contained in these follicles is pale-staining, vacuolated, or foamy and can often be seen adhering to the cilia. A few masses of apparently normal colloid may still be present in the center of the follicle, and this fact suggests that a degeneration process is going forward. These follicles

may open from one to another, and the contents appear to flow through the opening (fig. 3). The tracheal glands have ciliated lining cells which morphologically resemble these thyroid cells; but in the many sections examined, no connection was ever seen between the two, and they were always widely separated. The only description found of such cells in mammals is by Bacelar (*3*), who speaks of mucous and ciliated cells of the thyroid and shows figures of such cells in the rabbit and newborn cat. He writes:

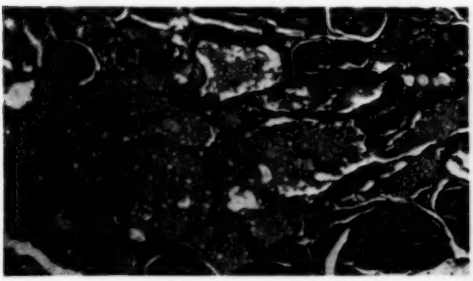

FIGURE 3.—Type of follicle in which ciliated cells are found. Hematoxylin and eosin stain. ×105.

> Concerning cilia, I have never found any allusion except the legend of a figure showing a ciliated vesicle in the work of Champy. This silence astonishes me greatly because mucous and ciliated cells are not so rare as that.

OBSERVATION FROM YOUNG ANIMALS

Single sections of thyroids were examined from 5 strain A mice, 11 weeks old, and no "degenerate" follicles and no ciliated cells were seen; but considering their infrequent occurrence in strain A animals, this is not surprising. Serial sections were then made of the thyroids from 10 newborn strain C3H mice, and a number of ciliated cells were seen. These cells were always found among the lining cells of small cysts or vesicles which contained a faintly acidophilic, granular, or vacuolated substance that differed from the deeply stained homogeneous colloid of the normal follicle. They were usually centrally located and were surrounded on all sides by the usual thyroid tissue. When traced serially, they sometimes branched but always ended blindly. One possibly significant observation was an apparent opening into a normal follicle, in which the colloid appeared to be breaking up into small masses (fig. 4). No connection to the trachea could be found.

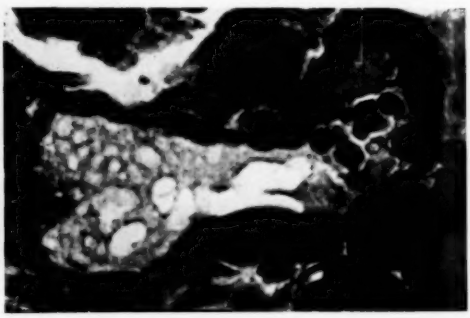

FIGURE 4.—Colloid in upper right follicle appears to be breaking up and passing to large central follicle containing vacuolated colloid. A ciliated cell in lower right corner of this follicle. Hematoxylin-and-eosin stain. ×13.0.

DISCUSSION

The most reasonable explanation for these ciliated cells is that they are persisting embryonic structures remaining from the pharyngeal epithelium from which the thyroid is developed. Such ciliated epithelium may be encountered in a thyreoglossal duct or in a thyreoglossal cyst in the human being; small cysts with some ciliated cells in the wall are recorded and have been seen in the thymus of the mouse, an organ having a similar embryologic history. Another possibility, however, is that these ciliated cells are functional and that the colloid of the normal follicles may become more fluid and be passed on into these abnormal follicles. It is even possible that the ciliated cells are present in follicles with normal colloid but cannot be identified except when the character of the colloid changes.

Whatever interpretation is made of these ciliated cells, this observation may be of interest in the controversy regarding the absorption of colloid, for Williams (*4*), in discussing the recognition of the thyroid as an "exocrine gland modified for endocrine secretion" observes:

> This would receive additional support from the finding in some lower forms of cilia on the distal ends of thyroid cells.

In a later paper (*5*) Williams studied the vacuoles in the colloid of the thyroid follicles of the isthmus in living mice by a method of transillumination. His mice apparently were not an inbred strain and are distinguished only as "white mice." No ciliated cells were mentioned. He discussed the ways in which vacuoles were formed and their probable significance.

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GROWTH RATE AND DEVELOPMENT OF TUMORS INDUCED WITH ULTRAVIOLET RADIATION

By HAROLD F BLUM,[1] formerly *senior pharmacologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

It hardly needs to be said that the rate of development of a tumor must be dependent upon the rate of tumor-cell proliferation, and of the original number of tumor cells, or, if carcinogenesis is a continuous process, on the rate of addition of new tumor cells. Such a generalization will scarcely be questioned, since it must apply if a tumor cell is a new type of cell as postulated by some, or is an ordinary tissue that has "escaped" as others believe. Many questions arise, however, and many differences of opinion appear, when one attempts a more specific analysis of tumor development. Since measurements have been largely lacking, it has been possible to maintain with about equal justification (1) that a tumor arises suddenly after a prolonged period of action of a carcinogenic agent and then grows without restraint, or (2) that the tumor is built up by the slow addition of tumor cells with proliferation playing only a minor part, or (3) that the carcinogenic agent brings about an increase in rate of proliferation of tumor cells. These possibilities, and others, have been directly expressed or tacitly implied by various investigators.

The present studies were directed toward evaluation of the importance of the rate of tumor cell proliferation in the over-all tumor development process.[2] Although, strictly, the findings apply only to tumors induced by ultraviolet radiation, some of them have general application.

EXPERIMENTAL RESULTS

MEASUREMENTS OF RATE OF TUMOR GROWTH

The material studied consisted of a series of 225 tumors of the ear of male strain A mice. All the tumors were induced by exposure to ultraviolet radiation according to the technique used in our earlier studies (*2*). The only experimental conditions which varied within this group were the dosage and the schedule of the exposures.

The growth rates were estimated according to the method described in a previous paper (*3*). This method is based on the equation for exponential growth:

$$\frac{\log V_2 - \log V_1}{t_2 - t_1} = K \qquad (1)$$

in which V_1 and V_2 are the volumes of the tumor at times t_1 and t_2, respectively. K is then an index of the relative increase in tumor volume during the interval between t_1 and t_2. K may be estimated from measurements of 1, 2, or 3 diameters of the tumor as outlined in the aforementioned paper (*3*); the more diameters measured the more reliable should the estimates be. Tumors of the ear, being well exposed, present favorable material for such measurements, and in these studies it was possible to base all the estimates of K on measurements of 3 diameters. In addition to the 225 tumors on which measurements are recorded, 28 others in the same series were examined and judged too irregular to measure, or in a few instances were measured and the measurements discarded because of their obvious unreliability.

In figure 1 are shown growth curves for a number of tumors which were measured at intervals over periods as long as 2 months. In this figure the logarithm of the tumor volume, estimated from the three measured dimensions (*3*), is plotted against t. This plotting would yield a straight line if equation (1) held throughout the course of the measurements. Obviously this is not the case, for in most instances the slope of the line decreases with time, indicating that the growth rate falls off progressively

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[2] The term "development" is used throughout this paper to include all changes which may lead ultimately to the appearance of a tumor. (See (*1*).)

as the tumor increases in size. Actually it would be impossible for a tumor to maintain a constant growth rate for more than a limited time; some restriction must be placed on tumor growth sooner or later by the failure of nutrient supply if not by other factors.

These curves give some idea of the inaccuracies which may enter into such measurements. In a few instances, it appears that the tumor has actually lost volume between measurements. This occurs, however, when measurements are separated by short intervals, or in small tumors, conditions under which errors in measurements are most likely to affect the curves; and these apparent decreases in growth rate probably represent inaccuracies of the estimates of volume rather than regression of the tumors. (See below.)

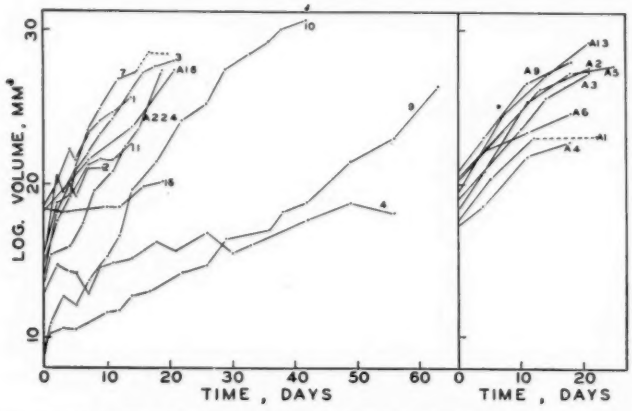

FIGURE 1.—Growth curves for some selected tumors. The dotted lines indicate that the measurements are uncertain due to erosion.

The slopes of the curves represent values of K in equation (1). It is obvious that K is not constant throughout the growth period, but that the slope of a given curve does not change radically in most cases. For example, the curve for a slow-growing tumor, such as No. 4, maintains a gradual slope throughout a period of 2 months, whereas the curve for the fast-growing tumor, No. A224, maintains a steep slope throughout its course. It seems, therefore, that K may be used as an index of tumor growth for comparison between tumors if comparable portions of the curve are used. There being no definite criterion for selecting corresponding regions of the growth curve, the week following selection of the tumors has been arbitrarily chosen. The criterion for selection was the attainment of a given tumor size (*4*), as has been the practice in all our previous studies; the average volume at this time was approximately 60 mm.³. Thus K, as recorded in the following data, represents the relative growth rate for the week following selection, and in most cases is based on only two measurements, one at the beginning and one at the end of that week. In a few cases the interval between measurements was from 5 to 9 days.

Of the 225 tumors for which K was estimated, 152 were judged to be "smooth" tumors, i. e., they were reasonably smooth in contour and did not markedly alter their shapes as they increased in volume. The remaining 73 tumors were more or less irregular, or in some cases became eroded before the necessary measurements were obtained. Although the former group should yield the most reliable estimates of K, no important difference between the two

groups was apparent. The mean value of K for the smooth tumors was 0.0517, t being measured in days (equation 1). The standard deviation for this group was 0.0210. The mean value of K for for the irregular group was 0.0487, the standard deviation 0.0258. The greater standard deviation for the irregular group might be expected, since the estimates of K are probably less reliable. However, the difference of the means is not significant ($P=0.3$ to 0.4), and it is probably justifiable to treat the smooth and irregular tumors as a homogeneous population. Figure 2 shows the distribution about the mean for both groups; although both are somewhat skewed toward lower values of K, the distribution approaches normal.

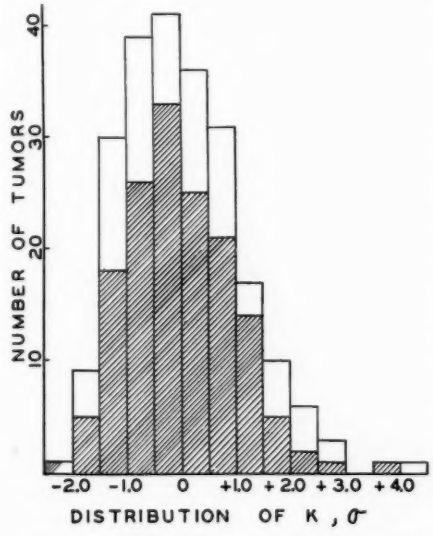

FIGURE 2.—Distribution of K. The shaded areas represent the smooth tumors, the open areas the total. Comparison is made in terms of standard deviation.

On the basis of the mean and standard deviation of K, it may be estimated that less than 1 percent of tumors of this size would regress, i. e., show negative values of K if normal distribution is assumed. This agrees with a general impression gained from observations of several hundred tumors under similar experimental conditions, among which no clear-cut instance of regression of gross tumors was noted.

In assuming that either the smooth or total groups represent homogeneous populations, certain factors need to be considered as regards their possible influence on the following analysis of the data. One of these concerns the relationship between K and the volume of the tumor, since the curves in figure 1 indicate that the growth rate usually falls off as the volume of the tumor increases. Actually, if the tumors are grouped according to volume at selection, K shows a slight but not very clear cut trend toward greater values for smaller tumors. Examination of the mean volumes of the tumor groups listed in tables 1 to 4 have shown that this factor has no important effect on the conclusions reached.

Histopathologic examination of tumors occurring under similar experimental conditions has shown the existence of several types (*4*, *5*). One type predominates, however, which consists of sarcomas of spindle-cell or more rarely of polymorphous-cell type. These probably originate for the most part from connective tissue though in some instances they may have their origin in muscle elements. This histologic type was present in 94 percent of the gross tumors examined, and although accompanied by squamous-cell carcinoma in 22 percent they probably formed the greater portion of the tumor in most cases. Squamous-cell carcinomas not accompanied by sarcomas and infrequent miscellaneous types comprised 6 percent of the total tumor population. There is some evidence suggesting that the carcinomas grow more slowly than the sarcomas (*5*). It is clear that the tumor population on which values of K were determined was not strictly homogeneous with regard to tumor type, and this may have influenced the distribution of K to a limited extent. However, since one type of tumor predominated to such an

extent, the group can safely be treated as a homogeneous population for statistical purposes.

TUMOR GROWTH AND DEVELOPMENT TIME

One common assumption with regard to tumor development is that the tumor cell, once it has been created by the action of a carcinogenic agent, proliferates "autonomously" at its own essential rate which is more rapid than that of its fellows. The growth of a tumor composed of cells proliferating at a constant rate is described by the equation:

$$\log \frac{N_2}{N_1} = \mathrm{K}t \qquad (2),$$

where N_1 and N_2 are the numbers of cells at the beginning and end of time interval t, and K is a constant. This equation has essentially the same form as (1), and if the assumption is made that the volume of the tumor is at all times proportional to the number of cells, K has the same numerical value as K. Thus K may be used as a measure of the rate of cell proliferation, if it can be assumed that no appreciable number of cells is destroyed or added by processes other than proliferation, as well as for the growth rate of tumors.

If the tumor were growing without let or hindrance at its own essential rate, some relationship between rate of tumor growth and time of appearance of the tumors might be anticipated, since faster growing tumors would be expected to appear earlier. Our data permit the examination of this possibility.

The term "tumor development time" may be applied to the period which elapses between the first application of a carcinogenic agent and the appearance of a tumor. For quantitative studies the term "individual development time" and the symbol t_d have been used to designate the time which elapses between the first exposure of an animal to ultraviolet radiation and appearance of a tumor of a given volume (about 60 mm.^{3}) (*4*). The values of t_d vary widely among the 225 tumors on which growth rates were obtained (91 to 351 days). The possible relationship between t_d and K is examined in table 1, where the tumors are arranged in groups of convenient size with respect to t_d. The mean values of K for the various groups do not differ significantly, nor is there evidence of a trend. Thus, there is no indication that development time and tumor growth rate are correlated.

TABLE 1.—*Growth rates of tumors with respect to individual development time,* t_d

t_d (in days)	Number [1] of tumors	Mean value of K
91–150	34	0.0429
150–175	67	.0534
175–200	57	.0547
200–250	47	.0497
250–300	11	.0502
300–351	9	.0462

[1] In this table and in tables 2, 3, and 4, only values for the total tumor group are given. If the smooth tumor group is treated in the same way, the same conclusions are reached.
(See p. 561)

This is, perhaps, not a reliable index of the relationship between growth rate and appearance of tumors; since all the animals were not treated identically but came from numerous experimental series among which the average t_d varies. All the mice in a given series (26 to 52) were given the same exposures to ultraviolet radiation, but the different series were treated differently in this respect. Within such series t_d varies widely, but in an orderly manner (*6*, *7*). In table 2, the tumors are arranged in groups according to the order of their appearance within the experimental series. Thus, the group designated 0 to 10 percent includes the first 10 percent of the tumors to appear in each series, the group designated 11 to 20 percent includes the next 10 percent to appear, etc. The mean values of K for the different groups do not differ significantly, and there is no evidence of a trend. Hence, no correlation between tumor growth and order of appearance is revealed by this arrangement of the data.

TABLE 2.—*Growth rates of tumors with respect to order of appearance*

Order of appearance (percent of tumors)	Number of tumors	Mean value of K
0–10	16	0.0380
11–20	27	.0593
21–30	23	.0535
31–40	26	.0494
41–50	25	.0459
51–60	36	.0496
61–70	22	.0656
71–80	23	.0488
81–90	12	.0493
91–100	15	.0503

Mottram (*8*) found that for a limited number of tumors examined the growth rates were greater in those that appeared earlier. On the other hand, Brues, Wiener, and Andervont (*9*) and Shimkin and Bryan (*10*), who studied chemically induced tumors, found no correlation between rate of tumor growth and time of appearance within a group receiving the same experimental treatment, findings in agreement with ours. Both Brues, Wiener, and Andervont and Shimkin and Bryan found that the growth rates of chemically induced tumors vary with the carcinogen and with the dose.

If the tumors were growing "autonomously" according to an inherent proliferation of the cells of which they are composed, it would be reasonable to expect that the tumors which grow most rapidly would appear first within the experimental series. Since this does not occur, as shown by the arrangement of the data in table 2, it would be necessary to assume that the growth period constitutes only a negligible part of the development time in order to rationalize the hypothesis of "autonomous" tumor-cell proliferation, and this possibility is ruled out on other grounds (table 5 and accompanying discussion). However, the possibility that tumor cells are constantly tending to assume an essential growth rate but are subject to control by the tissues is not ruled out. This hypothesis is discussed later.

TUMOR GROWTH RATE AND AGE

Development time increases significantly with the age of the mice (*7*). For this and other reasons, one might anticipate that the rate of tumor growth would vary with the age of the animals, but mean values of K for various age groups (table 3) do not differ significantly or suggest a trend.

TABLE 3.—*Growth rates of tumors in mice of different ages*

Age of mice (in weeks)	Number of tumors	Mean value of K
25–32	21	0.0479
33–35	45	.0494
36–38	49	.0540
39–41	56	.0517
42–44	28	.0526
45–55	18	.0490
56–61	8	.0422

TUMOR GROWTH RATE AND RECENCY OF EXPOSURE

In some of the experimental series from which the growth-rate data were obtained, the exposures to ultraviolet radiation were discontinued before the tumors appeared. If persistent effects on growth result from exposure to ultraviolet radiation, the growth rates of tumors of animals exposed regularly until the tumors appear might be expected to differ from those of animals not exposed for some time previous to that event. The arrangement of the data in table 4 was made to test this point.

TABLE 4.—*Growth rates of tumors with respect to time after last exposure to ultraviolet radiation*

Days after last exposure	Number of tumors	Mean value of K
0	132	0.0500
7	18	.0492
15	20	.0578
22–29	19	.0530
37	13	.0572
75 to 175	15	.0436
192 to 261	8	.0516

The most common procedure in our experiments was to expose the mice to ultraviolet radiation at regular intervals, usually 5 days per week, up to the time the tumor reached the size for selection. Tumors induced in this way

form the first group in table 4, comprising the category, zero days after last exposure. The value of K was determined during the week following, according to the usual procedure. The remaining tumors are grouped according to the time from the last exposure, which in some cases represents several months. The mean values of K for the various groups lie within the limits of chance variation, and no trend toward higher growth rates for tumors recently exposed to ultraviolet radiation is displayed. Hence, there is no evidence of persistent effect on the growth rate. Shimkin and Bryan (*10*) observed that a single dose of methylcholanthrene affects the average growth rate of tumors appearing weeks later, but this they explain as probably due to persistence of a trace of the carcinogen in the tissues which continues to bring about the formation of new tumor cells.

VARIATION OF GROWTH RATE DURING DEVELOPMENT

The evidence presented in the preceding section indicates that at about the time the tumor reaches a volume of 60 mm.^{3}, the average volume at selection, its growth rate bears no relationship to its development time or to the experimental procedure that has been followed in its induction. This evidence is based on mean values of K for groups of tumors. Further evidence that growth rate is independent of such factors is found when the growth rates of individual tumors are considered, as in table 5. In this table certain estimates have been made for individual tumors having a wide range of values of K. All are chosen from among those represented in figure 1 for which growth rates were followed for some time. Reference to that figure will show that the growth curves for these tumors are reasonably straight lines as plotted, i. e., they follow equation (1) fairly well during the period studied. The K values used in table 5 are those measured during the week following selection.

TABLE 5.—*Calculated data for selected tumors*

Tumor No.	K	Complete cell-division cycle	Development time t_d	Estimated size at first exposure [1]	Time after last exposure	Estimated size at last exposure [1]
		Days	*Days*	*Cubic millimeters*	*Days*	*Cubic millimeters*
16	0.0011	270	205	36	0	.....
A16	.0482	6	131	3×10^{-5}	0	.....
3	.0905	3	175	1×10^{-14}	0	.....
4	.0150	20	237	2×10^{-3}	150	3×10^{-1}
10	.0440	7	314	1×10^{-12}	219	1×10^{-5}
A224	.0885	3	354	6×10^{-31}	175	2×10^{-14}

[1] All the calculations are based on the assumption that growth rate remains constant throughout the development time.

Calculations show that the assumption that constant growth rate is maintained throughout the development time is untenable. In column 3 of this table the time occupied in an average complete cell-division cycle is given for the corresponding values of K. Obviously these rates of cell division could not have been maintained throughout the development time in all cases.

The volume at the first exposure of the animal to ultraviolet radiation has been estimated (table 5) for each of these tumors on the assumption that the growth rate represented by K was maintained throughout the development time. On the basis of this estimate, tumor 16 should have been clearly visible at the beginning of the experiment. This was not the case, and it is necessary, therefore, to assume that this tumor grew much more rapidly at some time earlier in its development than at the time of measurement. The volume of a tumor cell may be estimated to be about 10^{-6} mm.^{3}, and reference to the table shows that on the basis of the assumption followed, there could not have been a single tumor cell present in tumors 3, 10, and A224 at the beginning of the experiment. In contrast, if our assumption were true, a great many tumor cells must have been present in tumors 16 and 4 at the beginning of the experiment, while tumor A16 might or might not have consisted of a single cell, depending upon the accuracy of the estimate of tumor-cell

volume. This is not a very consistent set of values. The discrepancy is emphasized even more in the case of tumors 4, 10, and A224, in which the exposures were discontinued long before the appearance of the tumors. Estimations of the volumes of these tumors at the time the exposures were discontinued, based on the assumption of constant rates of growth represented by their respective K values, are given in table 5. According to these estimates tumor 4 was made up of many cells when the exposures were discontinued, while tumors 10 and A224 were not present as even a single cell.

No further elaboration of the point seems necessary. The fact is obvious that the growth rate exhibited by a tumor of measurable size is not an accurate index of its growth rate prior to that time. In some cases growth must be slower, in some cases faster, in stages of tumor development prior to the time the tumors become grossly detectable. This indicates that tumor growth is determined largely by factors other than the essential proliferation rate of the tumor cells. From this it may be inferred that the environment, i. e., the tissues, exerts control on the tumor at all times.

EARLY EVENTS IN TUMOR DEVELOPMENT

In an earlier paper (*1*), experiments were described in which the regular schedule of exposures was interrupted for a period of 1 month and then continued until the tumors appeared. These experiments did not involve a great many animals, and the schedule was otherwise varied, so that some uncertainty was involved in interpreting the results. A more extensive experiment was therefore set up. The data summarized in table 6 furnish information regarding early stages of development. All the animals were given a dosage per exposure of 2.0×10^7 ergs per square centimeter of mercury arc radiation of wavelengths shorter than 3200A. The intensity of the radiation was 3.3×10^4 ergs per square centimeter per second. (See (*2*) for details of the measurement of the dosage.) The exposures were given on 5 days per week except during a rest period of 30 days, which was introduced in most of the experiments. As in all our previous experiments the animals were male strain A mice.

TABLE 6.—*Effect of a rest interval on tumor-development time*

Group	Number of animals at 50-percent tumor incidence	First-exposure period	Rest period	Time to 50-percen tumor incidence, t'_{50}		Difference from expected [1]
				From beginning of first exposure	From end of rest period	
		Days	*Days*	*Days*	*Days*	*Days*
DK, C, G, O	89	154	None	154		
DI, DJ	52	71	30	169	68	−15
DG, DH	52	47	30	180	103	− 4
DE, DF	52	29	30	181	126	+3
DC, DD	52	17	30	190	143	+6
DA, DB	51	5	30	197	162	+13

[1] Expected time, if no further development or regression occurred during the rest period, would be 184 days, i. e., the 154 days required when there is no rest period, plus the 30-day rest period.

In only 1 series of 26 mice (DK) were the exposures continued without a rest period until the appearance of 50 percent tumors. The time to 50 percent incidence, t'_{50},[4] did not differ significantly from values obtained from earlier experiments in which the same dosage was used (*7*), and the results have been

[4] t'_{50} may be regarded as t_d for an "average" tumor (*6*, *7*).

pooled to obtain a more reliable value for the "control" conditions. This group is represented in the first line of table 6; the value so obtained for t'_{50} is 154 days.

In the remaining 5 series, each consisting of 52 animals at the beginning of the experiment, a rest period was introduced. The exposures were carried out at the regular schedule of 5 days per week for a given period which varied from 5 to 71 days in different experiments; this is designated first exposure period in table 6. This first-exposure period was followed by a 30-day rest period, after which the regular schedule of 5 exposures per week was resumed and continued until 50-percent tumor incidence was reached.[5]

The data show clearly that changes leading to the development of tumors can take place during the first exposure period and be carried over the rest period, that is, complete recovery does not take place during the rest period. In column 5 of table 6 the total time from the beginning of the exposures to the appearance of tumors, including the rest period is given. In all cases this is greater than the 154 days required when no rest period was given. In column 6 the number of days required after the rest period is shown. In the case of the shortest first period, 5 days, the time following the rest period is greater than the 154 days required when no rest period was given; in all the others, it is less. From previous data (*7*, *8*), it may be estimated that about 10 days represent a significant difference from the 154-day value ($P=0.05$). The increase of 8 days (from 154 to 162) which is shown by the group receiving a 5-day first exposure is not significant and hence may or may not indicate that tumor development is slightly delayed by such a short period of pre-exposure. However, the reduction for the group which received a first exposure of 17 days is significant (154 to 143 days), as is the case for all the groups receiving longer first-exposure periods. Thus, there can be no doubt that within a period of 17 days changes take place in the direction of tumor development, which persist to at least a certain extent over a period of 30 days during which the carcinogenic agent is not applied.

It may be pointed out that the primary carcinogenic agent here used, ultraviolet radiation, is completely absent during the rest period. This is a great advantage for such studies over the use of chemical carcinogens, the action of which may persist for some time. As has been shown earlier in this paper, there is no indication of a persistent effect on growth rate after exposure to ultraviolet radiation ceases.

This experiment gives further information regarding the nature of the changes in early stages of development. If it be assumed that changes occur during the first-exposure period which in the ensuing rest period neither develop further nor regress, t'_{50} should be increased in all cases by the length of the rest period. That is, since 154 days are required to reach 50-percent tumor incidence when no rest interval is given, 184 days should be required when a 30-day rest period is interposed. Reference to the values in column 5 of table 6 shows that in some groups the time required to reach 50-percent tumor incidence is less, in some more, than 184 days.

The differences are shown in column 7, which reveals an orderly sequence with regard to the length of the first-exposure period. In the case of the longest first-exposure period (group DI, DJ) 50-percent tumor incidence is reached 15 days earlier than predicted on the foregoing assumption, in the case of the shortest exposure period (group DA, DB) 50-percent incidence is reached 13 days later than predicted. The groups which are intermediate between these extremes with regard to the length of the first-exposure period arrange themselves in an orderly sequence of magnitude and sign with reference to divergence from the predicted value of 184 days for t'_{50}.

[5] In some instances the exposures were discontinued shortly before t_{50} was reached, but previous experience has shown that this should not affect the results appreciably.

Under these conditions, significance is represented by a difference of about 12 days. The extreme values in column 7 exceed this difference, and the orderly arrangement of the intermediate values follows a definite trend. In these experiments the time-incidence curves (log t_d vs probit, (6)) may be somewhat distorted, the introduction of the rest period affecting some parts of the curve more than others according to the conditions. The mid-portions of the curves appear less affected than the extremes, but nevertheless the values of t'_{50} must be regarded as somewhat less accurately established than in the experiments on which the estimates of significance are based (6, 7). However, the orderly sequence displayed in column 7 lends confidence to the validity of the relationship described.

If the foregoing interpretation of the data is correct, certain conclusions follow. It is evident that developmental changes take place during the first-exposure period which either advance or regress during the rest period that follows, depending upon the length of the first-exposure period. Thus presumably in the case of short first-exposure periods, development has not gone far, and regression dominates during the rest period. This would seem to be the case when the first-exposure period is less than about 29 days. On the other hand, when the first-exposure period is about 47 days or longer, a self-proliferative process seems to overcome the regressive process so that development actually advances during the rest period. It appears that an exposure period of between 29 and 47 days is required before the average tumor establishes itself and begins to grow.[6]

There is nothing here to suggest an induction period in the sense that a certain time or a certain number of exposures to ultraviolet radiation is required before the first tumor cell is produced (1). Rather, there seems to be a struggle between growth and regressive processes which determines whether the tumor shall become established and continue to grow. It is not difficult to picture a mechanism that will explain this. If tumor cells were produced at a constant rate because of the action of ultraviolet radiation and destroyed at a somewhat slower rate owing to action of the normal tissues, the tumor would grow so long as the exposures to ultraviolet radiation continued but would regress as soon as the exposures ceased. Since tumor cells would also be added by proliferation, however, a sufficient number of proliferating cells would eventually accumulate so that the tumor would continue to grow even though the action of the ultraviolet radiation were removed. If the exposures to ultraviolet radiation were discontinued before this stage was reached, the tumor would regress without gaining sufficient size to be observed grossly.

The concept of a struggle between growth and regressive processes might also explain the variability in growth rate illustrated by the data of table 5.

DISCUSSION

In a previous paper (1) the writer proposed a tentative explanation of the mechanism of development of these tumors, in terms of the then-existing data. This hypothesis assumed that tumor development was determined by three principal factors, the production of tumor cells by the action of ultraviolet radiation, the proliferation of these cells, and a control effect exerted by the tissues. The data presented herein suggest certain modifications which, however, need not alter the basic hypothesis.

[6] Our earlier experiments (1), like those herein described, indicate the existence of a self-proliferative mechanism after a certain point in development is reached but do not provide evidence for or against regression at earlier stages.

In the same study (1) it was found that no tumors appeared within 1 year following a continuous period of exposures lasting 12 days, whereas after a continuous exposure period of 74 days 50 percent tumors had developed 306 days subsequent to the first exposure. This suggests that the shorter period was insufficient for the tumors to become established, while the longer period was more than adequate for the average tumor. This might be considered as roughly confirmative of explanation given herein, although another possible explanation is suggested in the earlier paper.

Control by the tissues was considered to be largely an inhibiting effect on the rate of proliferation of the tumor cells. The possibility that such control might also include the destruction of tumor cells was suggested but was not emphasized because there was no evidence bearing on this point, other than certain histopathologic findings that could be otherwise explained. The data of the preceding section suggest that destruction of tumor cells owing to the action of the tissues occurs, and during early stages of development may be sufficient to prevent the establishment of a growing tumor.

It was assumed that the proliferation rate of the tumor cells is inherent in the cells themselves, that control exerted by the normal tissues slows the rate particularly in early stages, but that the tumor cells tend to escape and assume their own rate of proliferation. The measurements of growth rate described herein indicate a more dominant role for the control factor and show that the rate of proliferation of the tumor cells is not closely related to the overall rate of tumor development, as was at first expected. It is still possible to explain tumor development in terms of production of new tumor cells, proliferation of these cells, and control by the tissues including destruction of tumor cells; but proliferation becomes less directly involved in determining the rate of development, and the interrelations between the three principal factors appear more important. This complex of growth and regression may have a bearing on the mode of action of cocarcinogens and anticarcinogens and of endocrine control of tumor development.

Numerous aspects of the evidence suggest that tumor cells do not escape and grow autonomously, but that their proliferation is always under some degree of control by the tissues. This may be of considerable interest in regard to the cancer problem in general. In fact, it lends a ray of hope as regards the treatment of cancer, since it suggests that advantage may be taken of this control. There is, after all, support for this point of view, derived from the fact that regression of gross tumors may occur to a greater extent under some experimental conditions than under others (*11*). The present studies suggest that regression is a factor constantly present throughout tumor development even though it may not be obvious after the tumors reach gross dimensions. If this point of view is correct, it appears that more emphasis should be placed on study of the factors controlling the proliferation and fate of cells within tissues, both normal and cancerous.

SUMMARY

Measurements of growth rates of tumors induced with ultraviolet radiation are described.

The growth rates of the gross tumors are not correlated with the time required for their appearance but are the same for early and late appearing tumors. The growth rates are not correlated with the age of the animals, or with the recency of exposure to ultraviolet radiation.

Estimates based on individual tumors show that the growth rate does not remain constant throughout development and does not follow the same pattern in all tumors of the same type.

Experiments to determine the effect of interruption of the schedule of exposures on development time suggest that opposing growth and regressive processes determine the time at which a tumor becomes established so that it may continue to grow.

The general evidence indicates that the tumor cells do not escape and assume their own essential proliferation rates, but that rates of tumor growth are to a great extent dominated by the controlling influence of the tissues.

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REVIEW OF SERODIAGNOSTIC TESTS FOR CANCER[1]

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The results of numerous attempts to establish a serologic diagnostic test that would be of practical value in all types of cancer have been published. A review of this field of cancer literature reveals an amazing number and diversity of changes described as occurring in the blood of cancer patients. With recurring frequency, the originator of a test reports a high percentage of positive reactions with the serums of cancer patients while other investigators find the test of no practical value.

Even with our present limited means of therapy, the value of a diagnostic test that would reveal the site of a very small malignant growth is acknowledged by many. The questions that must be answered are whether all cancers in the early, operable stages are entirely local disturbances and whether the changes in the serums of cancer patients are detectable only after the chances of successful therapy are greatly diminished.

Before a discussion of a few of the older tests which have been used extensively, it might be worth while to describe some features of a therapeutically practical test. Since many pathologic conditions cause similar changes in the blood, there should be a marked degree of specificity to the test. However, in this connection it must be realized that other serologic tests, commonly used clinically, are not entirely specific and yet have practical value. With the Wassermann test for syphilis which Turner and Sternberg (*1*) applied to large numbers of army personnel, approximately 85 percent correctly positive reactions were obtained. These writers say that—

> biologic false positive serologic tests occur often enough after vaccination procedures and following acute febrile illness to constitute a serious diagnostic problem.

It would be of real clinical value to be able to differentiate tuberculosis and cancer when a palpable mass is present in the abdominal cavity, or when neurologic signs point to a lesion in the brain. The value of a test would be enhanced if it could be used postoperatively to indicate the absence of metastases or residual growths.

From the viewpoint of the biochemist, one rational procedure in devising a serodiagnostic test for cancer would be to search for products related to the altered metabolisms—carbohydrate, fat, or protein—of the neoplastic tissues. This procedure meets with difficulty since there is considerable dilution in the blood of any abnormal constituent of the tumor metabolism, which dilution might depend upon the ratio of tumor size to host size. While it is possible to demonstrate that the venous blood of a tumor contains more lactic acid than the arterial blood, this constituent would be metabolized normally by the tissues through which the blood flows before a sample is taken.

Many of the tests commonly used in European laboratories were developed before Warburg (*2*) introduced the concept of the altered metabolism of tumors, and many have thrived without biochemical interpretation. This is probably because many changes most evident in the blood of cancer patients are not directly related to the metabolism of tumor cells but may be the indirect effect of this metabolism upon the constituents of the blood. The tests that depend upon the instability of the serum protein fractions attest to this. The blood in other pathologic conditions undergoes similar changes, thus rendering many tests nonspecific for cancer.

Since recent reviews of the diagnostic test for cancer are available (*3–5*), only a few tests in which confidence has been placed by some investigators and which may serve as a basis for fu-

[1] Presented at a meeting of the staff of the National Cancer Institute, February 16, 1944.

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ture study will be discussed here. The tests may be roughly divided into three groups: (1) Those which measure the comparative concentrations of the constituents of the blood, or the chemical or physical characteristics dependent upon these constituents; (2) those which depend upon a specific antigen-antibody reaction; and (3) those which compare the proteolytic or lipolytic activities of the serums.

In the first group, the following tests are mentioned as representative:

(1) Accelerated sedimentation rate of erythrocytes of cancer blood (Walton (*6*)).

(2) Altered potassium-magnesium ratio of the serum during clotting (Links (7)).

(3) The proteose polarographic wave technique introduced by Brdička (*8–11*) which is reported to measure serum albumin fractions or a proteose fraction from albumin.

(4) A decrease in the "albumin A" fraction of serum in cancer (Kahn (*12–13*)).

(5) The meiostagmin reaction of Ascoli and Izar (*14–15*). A 10-percent solution of ricinoleic acid in methyl alcohol and normal saline is added to blood serum. In cancer serums a heavy flocculation occurs, but in normal serums there is only a slight opalescence.

(6) The increased rate of gelation of cancer serum upon addition of lactic acid (Kopaczewski (*16–17*)).

(7) The formation of a precipitate on addition of smaller quantities of citric or nitric acid to cancer serum in the presence of Lugol's solution. (Botelho's test as evaluated by Lavedan (*18*)).

(8) The spectrographic analysis of the proteins of the serum precipitated by sodium vanadate (Bendien (*19*)).

(9) The increased saponification and acid number of fats of cancer serum (Noël (*20*)).

(10) The neutral red test of Roffo, who postulates an increased globulin and lipoid content of the serum. A statistical study of the results of this test was reported by Gandolfo (*21*).

IMMUNOLOGIC TESTS

Immunologic tests postulate the formation of specific antibodies to the tumor tissues, and the specificity of the tests will depend upon the degree of foreignness, or the difference in chemical constitution of tumor and normal tissues. Water and fat-solvent extracts of tumor tissues have been used as antigens in the complement fixation and precipitin tests. Specific immune reactions have not been observed although it has been possible to demonstrate some degree of specificity by adsorbing out common tissue factors (*22–25*). Better results have been obtained lately where hydrolysates of tumor tissue were used as antigens (*26–27*). Few experiments have been reported with purified protein fractions of tumor tissue (Kidd (*28*)).

Hirszfeld and Halber (*29–30*) performed extensive clinical tests with the tumor lipids as antigens in complement-fixation reactions. While the serums of cancer-free patients did not show complement fixation with these lipid antigens, only 50 to 60 percent of the cancer serums reacted positively. These authors did not claim any clinical value for the test. It was improved by Lehmann-Facius (*31*), who found that the acetone precipitation of the benzene-soluble lipoids in extracts of cancer tissue yielded a phosphatide fraction which had marked specificity for cancer serum when used as an antigen.

ENZYMIC TESTS

Some of the tests that depend upon an altered enzymic activity of cancerous serums seem to offer the most promise. Two which are based upon lipolytic activity will be discussed first. The Freund-and-Kaminer test (*32*) originated in Vienna 34 years ago. It postulates that foods rich in animal fats change the flora of the intestines so that organisms are present which produce abnormal hydrolytic products of the fats. These substances enter the blood of the cancer patient and protect the cancer cells from lysis by the lytic enzymes. As the test is carried out, a suspension of cancer cells, usually from liver metastases, is incubated in a blood-counting chamber with the serum to be tested. The cells are counted before and after incubation. Normal serum cytolyzes from 25 to 60 percent of the cancer cells, while cancer serum cytolyzes 20 percent or less. The addition of one-half the amount of the cancer serum to the normal serum inhibits the lysis

of cancer cells. Very extensive studies have not succeeded in revealing the chemical nature of the lytic and antilytic factors (33). Freund and Kaminer (*34*) believed that the lytic factor is a dicarboxylic acid. Waterman (*35*) presented evidence indicating that a lipase and its activators are responsible for the carcinolysis. Christiani (*36–37*) reported that the antilytic factor is cholesterol-n-butyrate. But Lustig and Wachtel (*38*) showed that many organic compounds have antilytic activity.

The clinical value of the Freund-and-Kaminer test is limited (1) by the difficulty in preparing suitable suspensions of cancer cells for substrates, and (2) by the fact that patients must be selected who have not received radiation, sedation, or anesthetics, who are without hemorrhage or fever and are not alcoholics. Flazen and Wachtel (*39*) modified the test by substituting suspensions of adenocarcinoma cells from the mouse as substrates and, using only selected cases of cancer, obtained a high percentage of positive reactions. Benda and Kretz (*40*), of the General Hospital in Linz, reported that in a study of 1,434 cases, 91 percent of the cancer patients gave positive results and 84 percent of the noncancer patients gave a negative result.

Since the lytic action observed in the Freund-Kaminer test is probably enzymic, the studies of Bernhard (*41*) on the lipases of pathologic serums are of interest here. Bernhard first observed an atoxyl-fast lipase in the serum of patients with disturbed pancreatic function. It was during this study that he found a similar increase in this lipase activity in cases of cancer. Bernhard considered the lipase test especially valuable following surgery and radiation of neoplasms since a negative reaction quickly follows complete removal or destruction of the tumor. Increased lipase activity is found in several "precancerous" conditions: gastric ulcer, chronic mastitis, and prostatic hypertrophy, as well as in pancreatic and gall-bladder diseases.

Both the Abderhalden test and the Fuchs test measure an increased proteolytic activity of the cancer patient's serum, although the theoretical explanations for the tests differ widely. The experimental basis of the Abderhalden test was published about 1912, when Abderhalden reported that the parenteral administration of definite chemical compounds led to the presence in the blood, of specific enzymes capable of hydrolyzing these substances. Particularly, the administration of proteins gave rise to enzymes which specifically hydrolyzed these proteins, thus affording the body protection against them. This phenomenon of "protective" enzyme formation, which has not been entirely confirmed by others, was first given practical application in the diagnosis of pregnancy. In this case the "foreign" placental proteins entered the maternal blood causing the production of protective proteinases which specifically hydrolyzed the proteins. Likewise, according to Abderhalden (*42*), the foreign proteins of a tumor elicit the production of proteolytic enzymes which selectively hydrolyze only the same tumor proteins. In the test the substrates upon which the protective enzymes act are especially prepared proteins from tumors of various body sites. For example, the unknown serum is incubated separately with proteins from malignant tissues of the brain, liver, lung, etc. The serum of a patient with a carcinoma of the liver will hydrolyze specifically the liver-carcinoma protein. The degree of hydrolysis is determined by any of the several ways with which nonprotein nitrogen is measured. As with the immunologic tests, this test postulates the presence of chemically different proteins in the tumor tissues, which, because of their foreign nature, elicit an immune response, in this case a specific proteolytic agent. Biochemical and immunochemical studies of separated protein fractions of normal or tumor tissues have not progressed to a stage where the fundamental bases of these tests can

be judged. We know very little concerning the exact chemical structure of the tissue constituents upon which this type of immune reaction probably depends.

Abderhalden and his coworkers (*43*) report the correct diagnosis of thousands of cases of carcinoma. Probably because of the difficulty in preparing the specific substrates the test has not met with success in the hands of some investigators. If one assumes that the fundamental basis is sound, the test should achieve greatest accuracy if substrates of the identical cell type from which the cancer arose were selected. This selection requires a clear working knowledge of the embryologic relationships of normal and malignant tissue cells.

FUCHS TEST

In 1926 Fuchs (*44*) found that serum from cancer patients hydrolyzed fibrin from normal blood but not fibrin from cancer blood. Likewise, serum of normal blood hydrolyzed cancer fibrin but not normal fibrin. Notwithstanding the careful, microchemical, quantitative technique necessary to demonstrate the very slight hydrolysis which occurs, many investigators have confirmed this test. Up to 1934, according to Bing and Marangos (*45*), 5,000 tests had been reported in which 92 to 94 percent of correct diagnoses had been made. Within the last 10 years more than 25 different investigators (*4*) have used the Fuchs test and obtained 85 to 93 percent positive reactions with the serums of cancer patients and low percentages of false positive reactions.

Careful enzymic studies of this test were made by Wright and Wolf (*46*) at Cambridge, in which they found that the reaction follows the Schutz law, i. e., the velocity of the reaction is dependent upon temperature and within certain limits also upon the amount of substrate present. No studies were made of the hydrogen-ion concentration. Woodhouse (*47*) was able to diagnose correctly 50 of 53 malignant cases and 38 of 42 nonmalignant cases. He found that the Fuchs test was positive in external epitheliomas where the extent of the lesion was not great and suggested that the test should be a help in relatively early cases of internal cancer also.

In view of the apparent success of the Fuchs test, efforts should be made to reconcile the theoretical considerations of Fuchs concerning it with experimental evidence. Fuchs' hypothesis is that specific immune bodies are adherent to the fibrin and that they protect the fibrin from hydrolysis by the enzyme of its homologous serum. This hypothesis was further elaborated to include a differential test for species, in which the serum of one species could not hydrolyze its homologous fibrin (*48*). Van Der Scheer (*49*) was unable to obtain evidence of species differentiation by the Fuchs test. Fuchs (*50*) described an improved procedure for the test in which the dried trichloracetic acid precipitate of serum is used as the substrate instead of fibrin. This change might indicate that the postulated immune protective factor is associated not only with the fibrin but also with the entire protein fraction of the serum.

The proteinase activity of normal serum is very slight. The work of Oppenheimer (*51, p. 928*) indicated that the proteinases of blood were almost completely masked by adsorption on the serum proteins. Only slight shock is required to liberate the enzyme from the serum proteins since it is freed by organic solvents, dilution, or dialysis of the serum. It is quite possible that the change in stability of the cancer serum proteins already described may be a factor in liberating the proteinase which acts in the Fuchs and Abderhalden tests. While Oppenheimer believed the proteinase in serum to be trypsin, Kleinmann and Scharr (*52*) presented evidence that it is cathepsin.

It is interesting in this connection to recall that the Russians, who were among the first to establish blood banks, used the blood of persons who had met with sudden or violent death. If, in these cases, the blood was withdrawn

soon after death, it was found that although the clot formed it was dissolved in an hour or two and that no further tendency to clot was observed. The blood could be preserved in this state until needed for transfusion. Macfarlane (*53*) also found that in many cases the fibrin clot was partially dissolved after surgical shock. Mason and Evers (*54*) obtained the Fuchs reaction (increased proteolytic activity of the serum on dog fibrin) with the serum of dogs that had experimental uremia produced by bilateral ureteral ligation. Since there is this evidence that serums under varying conditions have enzymes capable of hydrolyzing the homologous fibrin, it is difficult to understand why cancer serum does not hydrolyze cancer fibrin. It is possible that some specific inhibitor other than the postulated protective immune bodies may play a role here. Cancer serums were reported by Reding (*55*) to have a high index of polypeptidemia, which may also be demonstrated by the proteose polarographic reaction. This increase in protein split-products in the cancer serum might tend to inhibit the forward movement of the proteolytic reaction. The inhibition of proteolysis may be due to a combination of factors, including the hydrogen-ion concentration of the digest containing fibrin and cancer serum.

A comparative study of some of these tests was made by Bernhard (*55*). Since his percentages of positive and false positive reactions agree closely with those of most of the other investigators, the results of his tests are reproduced here (table 1). It should be noted that none of the tests have been found useful by all investigators. To check the tests it is obvious that most of the cancer serums used have come from patients whose neoplasms had already been diagnosed clinically. It is, of course, in the much earlier stages of the disease that a serodiagnostic test is needed. With this in mind, Bernhard broke down the cases giving false positive reactions into four groups: (1) Normal; (2) precancerous, which included gastric ulcer, chronic cystic mastitis, and prostatic hypertrophy; (3) diseases of the pancreas and gall bladder; and (4) chronic inflammatory diseases. The significance of the false positive reactions in the so-called precancerous group can be clarified only by a careful, extensive study of the case histories of many patients in this group over a long period. To evaluate any of the tests as a means of the early diagnosis of cancer, it would be necessary to apply the tests at regular intervals to large groups of persons before and through the "cancer years." Then a study of the cancer cases that appeared would reveal how early the diagnosis of cancer could be made.

The recent developments in the diagnosis of cancer have been away from the general tests just described toward those tests that indicate a disturbance in organs with definite secretory functions or highly specialized activity. For instance, a tumor of the pancreas affect-

TABLE 1.—*Results of five different serodiagnostic tests for carcinoma* [1]

Test	Carcinoma			Carcinoma-free		Distribution of false positive reactions			
	Cases	+	−	Cases	+	Normal	So-called precancerous diseases	Gall bladder and pancreas diseases	Chronic inflammatory diseases
	Number	*Pct.*	*Pct.*	*Number*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*
Fuchs (fibrinolysis)	406	89.14	10.86	481	25.92	14.76	52.38	28.57	4.29
Waldschmidt-Leitz (polarographic)	169	86.99	13.01	553	22.50	15.87	30.15	23.80	30.18
Lehmann-Facius (complement-fixation)	38	71.06	28.94	124	37.90	25.53	19.15	21.27	34.05
Freund and Kaminer (cytolysis)	42	71.17	28.83	63	38.09	29.17	20.83	8.33	41.67
Bernhard (atoxyl-fast lipase)	466	84.77	15.23	713	30.51	9.23	43.07	30.77	16.93

[1] Data from Bernhard (*56*).

ing the islets of Langerhans may cause hyperinsulism with blood sugar changes which can be detected. Huggins and Hodges (*57*) found that marked increases in acid phosphatase activity of the serum are observed in prostatic carcinoma and that an accompanying increased alkaline phosphatase activity may indicate skeletal neoplasms.

Advances in endocrinology have led to specific biologic tests for cancer. In 1929 Zondek (*58*) first observed Prolan A in the urine of a man suffering from teratoma testis. Prolan A is the gonadotropic hormone of the anterior pituitary which is effective in Aschheim-Zondek test for pregnancy. Using the Aschheim-Zondek test with quantitative refinements, Ferguson (*59*) was able to diagnose teratoma testis in 117 cases, with an error of 8 percent. In the absence of pregnancy the Aschheim-Zondek test can also be used to diagnose chorionic carcinoma (*60*). The test is particularly useful in following the growth of possible metastases after surgical removal of these tumors.

The limited number of organs with potent secretions restricts the biologic diagnosis of cancer to a comparatively few sites, so that a general test for cancer is still needed. At present a few of the general tests appear to have a specificity equal to that of other clinical tests when the cases are carefully selected and the disease has progressed to a stage where it can be diagnosed clinically. But before any of these tests can be recommended for use in the early diagnosis of cancer, they should be improved and if possible applied to the blood of animals bearing spontaneous tumors. Further intensive biochemical and immunochemical research on the constituents of tumor tissue, and especially on the effects of these factors on the blood of the host may lead to new and more specific tests for cancer.

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INFLUENCE OF ENVIRONMENT ON MAMMARY CANCER IN MICE

By Howard B. Andervont, *principal biologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

During the course of an experiment in which female strain C3H mice lived alone or in contact with other mice, it was found that those kept in separate cages developed mammary tumor earlier than did their litter mates kept eight to a cage. This observation was confirmed in a series of investigations reported herein.

The mice were raised in this laboratory and were maintained on a diet of Purina dog chow pellets with an unlimited supply of water. These mice (*1*) are highly susceptible to the development of spontaneous mammary cancer when bred or when kept as virgins.

The cages were wooden boxes 28 cm. long, 18 cm. wide, and 15 cm. deep, and eight mice were housed in them without obvious overcrowding. The segregated mice lived in cages of the same type, divided into two compartments by a metal partition. Thus each segregated mouse occupied a space 14 by 18 by 15 cm., or just half the size of the cage. Sawdust was used as bedding, and the cages were cleaned twice weekly.

Three experiments were performed using virgin or breeding mice. Litters were always divided between segregated and nonsegregated animals. In the first experiment, the mice were isolated or placed eight to a cage when 4 weeks of age and were kept as virgins. In the second, they were also maintained as virgins but lived eight to a cage until they were 20 weeks old when the litters were divided among the segregated and nonsegregated groups. In the third experiment, breeding mice were isolated or kept eight to a cage at the time of weaning and were bred when 12 weeks old. A male was placed with each segregated female for 4 days. All litters were removed within 24 hours after birth.

The results of these experiments are summarized in table 1. Virgins isolated when 4 weeks old developed mammary tumor at an average age of 9.6 months, while their nonsegregated litter mates did so at an average age of 11.9 months. The only segregated mouse not developing a tumor lived 20 months, whereas four of the nonsegregated mice attained the same age and were free of tumor.

Virgin mice of the second experiment that were segregated when 20 weeks old developed tumors at an average age of 9.1 months. Tumors arose in their nonsegregated litter mates at an average age of 10.6 months. The only segregated mouse not developing a tumor survived 16 months; 3 of the nonsegregated animals lived to the same age and were tumor-free.

In the third experiment, tumors occurred in the segregated breeding mice at an average age of 7.8 months, while the nonsegregated litter mates showed tumors at an average age of 8.6 months.

In these experiments the average age at which tumors arose in nonsegregated animals was in accord with previous observations on this strain of mice, but the tumor incidences of 80.3 and 83.3 percent in the nonsegregated virgins were lower than the 97.4 percent incidence in the earlier study (*1*). All earlier data were obtained with mice kept eight to a cage because this has been the routine procedure in this laboratory.

The outcome of these experiments permits the conclusion that strain C3H mice living in isolation develop mammary tumors earlier on the average than do litter mates living eight to a cage. This is true of virgin mice isolated when 4 or 20 weeks of age as well as breeding mice segregated at the age of 4 weeks.

A survey of the literature has not as yet revealed a report of similar findings. Dr. L. C. Strong,[1] of the Yale University School of Medicine, states that he noted a correlation between the number of mice kept in a cage and the incidence

[1] Personal communication.

TABLE 1.—*Occurrence of spontaneous mammary tumor in segregated and nonsegregated strain C3H mice*

[Numerators=mice developing tumors; denominators=mice dying free of tumor]

Experiment No.	Group	Virgin or breeding	Age of mice when segregated	Number of mice	Age (in months)																	Incidence of mammary tumors	Average tumor age	Average age of mice dying without tumor
					5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	Total				
			Weeks																			*Percent*	*Months*	*Months*
1	Segregated	Virgin	4	58	1/0	5/0	5/0	10/0	9/0	6/0	13/0	2/0	1/0	2/0	1/0	1/0	----	1/0	0/1	57/1	98.3	9.6	20.0
	Nonsegregated	do	4	56	----	1/1	1/1	4/0	4/1	3/2	7/0	7/1	5/1	5/0	2/0	3/0	2/0	1/0	0/4	45/11	80.3	11.9	13.4
2	Segregated	do	20	31	----	----	4/0	6/0	5/0	3/0	6/0	4/0	1/0	1/0	----	0/1	----	----	----	30/1	96.8	9.1	16.0
	Nonsegregated	do	20	24	----	----	1/0	2/0	2/0	6/0	3/0	2/1	2/0	2/0	----	0/3	----	----	----	20/4	83.3	10.6	15.0
3	Segregated	Breeding	4	30	1/0	3/0	9/0	9/0	4/0	3/0	1/0	----	----	----	----	----	----	----	----	30/0	100.0	7.8	----
	Nonsegregated	do	4	25	----	4/0	1/0	6/0	6/0	6/0	2/0	----	----	----	----	----	----	----	----	25/0	100.0	8.6	----

and frequency of mammary cancer. He used boxes 5 x 6 x 11 inches. When no more than 5 mice lived in a box, the maximum incidence of breast tumors occurred; when 6 to 8 mice lived together, the age incidence was delayed; and when 10 to 14 lived together, mammary cancer was practically eliminated. Strong's observations included only breeding mice of strains C3H and A.

The present data, together with Strong's unpublished findings, show the influence of environment upon the occurrence of spontaneous mammary-gland tumors in certain inbred mice. Apparently the incidence and time of appearance of this neoplasm in a strain of mice should be determined by maintaining the animals in isolation if possible or by allowing small numbers to live together. Crowded living conditions or contact with other mice, or both, increase the average age for tumor development and, according to Strong, decrease tumor incidence.

The occurrence of mammary tumor in the mouse is controlled by at least three factors, namely, genetic susceptibility, an agent or agents in the mother's milk, and hormonal stimulation. Crowding or contact should not influence the genetic susceptibility or substances obtained in milk but may interfere with hormonal stimulation. An exploratory investigation in which vaginal smears were obtained from segregated and nonsegregated littermate strain C3H virgin mice indicated that estrous cycles in the segregated animals occurred earlier, were more frequent, and lasted longer than in those living eight to a cage. This suggests that the results recorded here were due to environmental conditions exerting an influence upon hormonal stimulation.

To date, a report on the effect of isolation or crowding upon the estrous cycle in the mouse has not been found. Long and Evans (*2*) reported that rats kept in isolation or living together in small numbers showed no difference between ovulation periods. Small num-

bers of mice living in ample quarters may develop tumors as early as those living alone. White and Andervont (*3*) used 42 virgin strain C3H mice which, starting at 4 to 5 weeks of age, lived 2 in a cage; 41 developed mammary tumors at an average age of 8.36 months.

In the present work the effects of diet, temperature, activity, and other possible factors were not studied. There was no obvious difference in the general appearance of segregated and nonsegregated mice. One group of virgins was weighed when they were 7 months old, and the average weight of 33 segregated and 28 nonsegregated was 26.7 and 25.8 gm., respectively. This difference is not considered significant. Shimkin and Grady (*4*) found that strain C3H male mice living in individual cages consumed more food in proportion to body weight than did others kept five to a cage. Further work is necessary to establish the reasons for the different average ages at which tumors arise in segregated and nonsegregated strain C3H female mice.

The primary purpose for placing this work on record is to show that in studies on the occurrence of mammary tumors in mice the cages of control and of experimental animals must contain equal numbers of mice.

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EFFECT OF TWO AZO COMPOUNDS WHEN ADDED TO THE DIET OF MICE

By HOWARD B. ANDERVONT, *principal biologist*, JULIUS WHITE, *senior biochemist*, and JESSE E. EDWARDS, *research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service*

A previous experiment (*1*) showed that *o*-aminoazotoluene when moistened with glycerol and administered subcutaneously to inbred mice and their hybrids induced hepatic changes, hepatomas, pulmonary tumors, and hemangio-endotheliomas. For all inbred strains and hybrids, female mice were more susceptible than males to induced hepatic changes and hepatomas. Another experiment (*2*) was designed to ascertain whether subcutaneous administration of the compound dissolved in olive oil gave similar results and whether mice exhibit a sex difference in susceptibility to hepatic changes induced with *p*-dimethylaminoazobenzene, which produces hepatic lesions (*3*) in rats. The results of this study showed that the administration of *o*-aminoazotoluene evoked hepatic reactions, hepatomas, pulmonary tumors, and hemangio-endotheliomas, as well as subcutaneous fibrosarcomas, whereas *p*-dimethylaminoazobenzene when dissolved in olive oil produced only fibrosarcomas at the site of injection and when moistened with glycerol failed to produce any lesions.[1]

At the time the subcutaneous injections of olive-oil solutions were started, another experiment was begun in which *o*-aminoazotoluene and *p*-dimethylaminoazobenzene were added to the food. This investigation also was undertaken to determine whether mice exhibit a sex difference in susceptibility to the compounds and whether the compounds induce a variety of tumors when administered orally. This and a subsequent experiment are reported herein.

[1] These observations with *p*-dimethylaminoazobenzene were based upon mice kept 1 year after receiving the first injection. However, eight strain C males and eight strain C females that received the olive-oil solution and five strain C females that received the glycerol-moistened compound were kept to see whether they would develop lesions at a later date. All lived 19 months after the initial injection, and none developed lesions.

MATERIALS AND METHODS

Strain C mice 6 to 8 weeks of age were used. They were derived from the colony maintained at the National Cancer Institute. All lived under similar environmental conditions and were given an unlimited supply of drinking water. The diet had the following composition:

	Percent
Casein (crude)	6.0
Cornstarch	50.0
Crisco	24.0
Sucrose	15.0
Cod-liver oil	1.0
Inorganic salts (*4*) (modified Osborne-Mendel)	4.0

To each 100 gm. of these constituents were added 5 gm. dried brewers' yeast, 0.5 gm. cystine, and either 0.06 or 0.03 gm. of the azo compound [2]; the amount used is given in the description of the individual experiments.

Necropsies were performed on all mice. The macroscopic appearance of the liver was noted, and a piece of each was kept for histologic examination. Other organs were examined for the presence of gross tumors. The occurrence of hepatic reactions and all tumors was confirmed microscopically. The gross and the microscopic appearance of hepatic lesions and tumors were similar to those described in the previous paper (*1*).

EXPERIMENTAL PROCEDURE

EXPERIMENT 1

During June 1941 male and female strain C mice 6 weeks of age were given the experimental diet. Eleven males and nine females ate the diet containing 0.06 gm. *p*-dimethylaminoazobenzene, while 12 males and 12 females consumed

[2] The compounds were obtained from the Eastman Kodak Co., and were purified by Dr. J. L. Hartwell, of the National Cancer Institute: *o*-aminoazotoluene (m. p. 102.5°–103° C., corrected) and *p*-dimethylaminoazobenzene (m. p. 117°–118°, corrected).

the diet containing 0.06 gm. *o*-aminoazotoluene.

The animals gained weight slowly and after 32 days were in poor physical condition. During the next 9 days, the diet containing the azo dyes was withdrawn, and the animals were fed Purina dog-chow pellets, the standard diet in this laboratory. This procedure was necessary throughout the experiment. The alternating dietary regimes, in days, were as follows:

Animals receiving *p*-dimethylaminoazobenzene:

Experimental diet	*Dog chow*
32	—
—	9
28	—
—	5
7	—
—	3
25	—
—	4
24	—
—	9
26	—
—	10
128	—

Animals receiving *o*-aminoazotoluene:

32	—
—	9
28	—
—	5
7	—
—	3
60	—
—	11
27	—
—	7
29	—

Three males and three females fed the *p*-dimethylaminoazobenzene diet for 178 days were killed, and none showed tumors or hepatic lesions. The remaining mice of the group, eight males and six females, were killed after consuming the experimental diet for 270 days. None of the males had a tumor or hepatic reaction; the livers of four females were cirrhotic in appearance, and one of them contained three hepatomas.

In previous experiments strain C mice received subcutaneous injections totaling 45 mg. of *p*-dimethylaminoazobenzene dissolved in olive oil (*2*) or 100 mg. moistened with glycerol (*1*), and none developed a hepatic reaction. The occurrence of hepatic lesions and hepatomas in females of this experiment indicates that this strain of mice is not completely resistant to the carcinogenic action of the compound. Other types of tumors were not found in any of these animals.

Mice fed the *o*-aminoazotoluene diet were killed after eating the experimental diet a total of 183 days. The necropsy findings are summarized in table 1. All showed hepatic reactions, but the females showed more pronounced lesions than did the males. Further, 11 females developed hepatomas as compared with 7 males. There were no significant differences between the sexes in their incidences of induced pulmonary tumors, but hemangio-endothelioma of the lungs occurred in 10 females and in only 2 males.

Although the results of the investigation indicated sex differences in susceptibility to *o*-aminoazotoluene administered in the food, it was deemed necessary to carry out another experiment to confirm the observation.

EXPERIMENT 2

During May 1942, 11 litters of strain C mice were separated according to sex, and when 8 weeks old they were fed the experimental diet containing *o*-aminoazotoluene. Since the equivocal findings of the first experiment may have resulted from an overdose of the carcinogen which masked a clear-cut sex difference in response, the amount of the compound in the diet was reduced to 0.03 percent. Thus, the daily intake of the compound was reduced to one-half that of the first experiment. To compare the results with those obtained previously when 100 mg. of the dye was injected subcutaneously (*1*), the amount ingested was limited to approximately 100 mg. for the females and 130 mg. for the males.

The animals were kept two in a cage, and their daily food consumption was ascertained for the first 70 days of the experiment. During this time the females ate an average of 1.5 gm. and the

TABLE 1.—*Occurrence of hepatic reactions and various types of tumors in strain C mice following ingestion of a diet containing o-aminoazotoluene*

Experiment No.	Number of mice	Sex	Azotoluene ingested [1]	Number of mice developing—					
				Hepatic reaction	Hepatoma	Pulmonary tumor	Hemangio-endothelioma		
							Lung	Interscapular fat	Peritoneal fat
			Milligrams						
1	12	F	165	12	11	11	10	1	
	12	M	220	12	7	10	2		
2	19	F	100	18	13	16	6		1
	17	M	130	7	1	10	2		

[1] Approximate amount.

males 2.0 gm. per day. After consuming the diet 100 days, the mice were in poor physical condition, and their food was changed to dog chow 1 day each week. Under this regime, the animals remained in excellent health.

After ingesting the experimental diet 157 days, 4 males and 4 females were killed. One female showed a slight hepatic reaction; all others were negative. The feeding was continued until the mice had eaten the experimental diet 223 days, when all survivors (17 males and 19 females) were killed and necropsied. The findings are given in table 1, which includes only those animals killed after consuming the experimental diet 223 days. The data in the fourth column of the table were obtained by a computation based upon the amount of food consumed during the first 70 days by the mice of experiment 2.

The outcome of the second experiment revealed that mice of strain C exhibit a sex difference in susceptibility to *o*-aminoazotoluene when the compound is added to the diet, but the results were not so clear cut in this respect as were those obtained following subcutaneous injection (*1*, *2*). This result, together with those of the first experiment, suggest that the addition of large amounts of *o*-aminoazotoluene to the diet or prolonged feeding may mask this difference.

As shown in table 1, females of strain C were more susceptible to induced hepatomas than were the males, but there was no pronounced sex difference to induced tumors of the lung. Sixteen of thirty-one females and four of twenty-nine males developed pulmonary hemangio-endotheliomas. This confirms earlier observations (*1*) that the females are more susceptible to the induction of this type of neoplasm.

When strain C mice received subcutaneous injections of *o*-aminoazotoluene (*1*, *2*), most of the hemangio-endotheliomas were found in the fat of the interscapular region, although some arose in the lung, ovary, and adrenal, as well as in the abdominal and subcutaneous fat depots. In the experiments recorded here, one tumor was observed in the interscapular, and one in the abdominal fat, but the lung was the chief site of origin for this type of tumor. Induction of hepatomas, pulmonary tumors, and hemangio-endotheliomas by oral administration of the compound is in harmony with results attending its subcutaneous injection, namely, that it is capable of producing a variety of tumors in the mouse.

Finally, this work also confirms earlier findings that in the mouse *o*-aminoazotoluene is a more potent carcinogen than is *p*-dimethylaminoazobenzene. However, the present work shows that the mouse is not completely resistant to the carcinogenic action of the latter.

SUMMARY

Diets containing *o*-aminoazotoluene and *p*-dimethylaminoazobenzene were fed to strain C mice of both sexes.

The former induced many hepatic changes, hepatomas, pulmonary tumors, and hemangio-endotheliomas, whereas the latter induced only a few hepatic reactions and few hepatomas.

Female mice were more susceptible than males to hepatic reactions, hepatomas, and hemangio-endotheliomas induced with *o*-aminoazotoluene. When orally administered, this compound elicited many pulmonary hemangio-endotheliomas.

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ADMINISTRATION OF 3, 5-CHOLESTADIENE AND DICHOLESTERYL ETHER TO MICE AND RATS

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INTRODUCTION

Development of the chemistry of chemical carcinogens was followed by the reports of several investigators who described experiments designed to demonstrate a possible relationship between dietary fats and gastric tumors. The possibility of some relationship became apparent as the structural configurations of the carcinogenic hydrocarbons were evolved. The structural similarity of these hydrocarbons and the sterols of animal and vegetable fats elicited considerable speculation which led to a series of investigations in several laboratories.

Roffo has repeatedly claimed that degradation products of cholesterol, or other sterols, obtained by irradiation or by heating, were responsible, in part, for the genesis of skin and gastrointestinal tumors in humans and in experimental animals. He described (*1*) the production of adenocarcinomas in the glandular portion of the stomachs and fusocellular sarcomas in the livers of rats that had been fed diets containing irradiated cholesterol and stated (*2*) that the feeding of heated animal fats led to the development of the same types of tumors in the same organs. In each of these reports the results were interpreted as due to oxidation of the cholesterol in the fat to a carcinogenic "oxycholesterol." Waterman (*3*) found 4 tumors, diagnosed as squamous-cell carcinoma of the forestomach, in 46 mice that had been fed cholesteryl oleate for nearly a year. Veldstra (*4*) prepared 3, 5-cholestadiene from cholesteryl oleate and furnished some of the hydrocarbon to Waterman, who fed it to mice. After 1 year, 4 papillomas of the stomach (site not clear) were found, 1 of which, it was claimed, showed infiltrating properties. Kirby (*5*) reported negative results of the degradation products of cholesterol heated at 270°–300° C. after feeding 20 mg. to rats daily for 2 years. Likewise, 3, 5-cholestadiene, 20 mg. fed daily for 18 months, elicited no malignant gastric lesions. Strong, as cited by Bergmann and Skau (*6*), obtained negative results when benzene or sesame oil solutions of 2,4-and 3,-5-cholestadiene were painted on or injected into strain CBA mice. Steiner, Steele, and Koch (*7*) obtained no tumors up to 18 months when cholesterol that had been heated to 200° and 300° was injected subcutaneously into mice.

In conjunction with the investigation of Morris, Larsen, and Lippincott (*8*), wherein heated dietary fats were fed to rats, a study of orally and subcutaneously administered 3,5-cholestadiene appeared to be warranted. The present paper is concerned with the effects of this hydrocarbon when fed to rats and when implanted subcutaneously, in the form of pellets, in rats and mice.

EXPERIMENTAL PROCEDURE

PREPARATION OF 3,5-CHOLESTADIENE

Preparation of 3,5-cholestadiene in quantity was effected by the dehydration of cholesterol in the manner described by Mauthner and Suida (*9*), and Eck, Van Peursem, and Hollingsworth (*10*), with minor modifications. Cholesterol (100 gm.), intimately mixed with anhydrous copper sulfate (100 gm.), was heated under reduced pressure in an ordinary distilling flask with the aid of a molten metal bath. The cholesterol-copper sulfate flux was maintained at 180° C. for about 30 minutes, or until foaming had ceased. The reaction mixture was cooled and partitioned between water and either chloroform or ethyl ether. The solution of the reaction mixture was washed with water, dried over anhydrous sodium sulfate, and the solvent removed completely under reduced pressure. The

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sirupy residue was dissolved in 2 l. of hot ethyl acetate and allowed to stand overnight at 0°. Dicholesteryl ether, which is one of the products of dehydration of cholesterol, crystallized out almost quantitatively under these conditions. This product, which usually amounted to about 15 percent of the reaction mixture, was filtered with suction and washed with cold ethyl acetate. This crude fraction was set aside for later study. The filtrate and washings were combined and concentrated to a volume of 800 cc. and again allowed to stand overnight at 0°. A very small amount of gelatinous material collected in the bottom of the flask and was found to be principally dicholesteryl ether. The supernatant solution was decanted, reduced in volume to 200 cc., and allowed to stand for several days at −10°. 3,5-Cholestadiene in the form of pale yellow prismatic crystals was deposited, and the yield was good. This crystalline fraction (m. p. 76°–78° C.) was filtered with suction and washed with small portions of cold ethyl acetate. It usually amounted to 50–55 percent of the reaction mixture. Further concentration of the mother liquor led to to the isolation of about 5 percent more crude material (m. p. 75°–77°). Joining of these crude fractions and recrystallization from ethyl acetate resulted in 3,5-cholestadiene (m. p. 79°–80°). The specific rotation ($[\alpha]_D^{22}$ in chloroform), with different batches, varied between −95 and −100, whereas slightly higher optical values have been reported (*4*, *10*). Stavely and Bergmann (*11*) established the identity of their dehydration product of cholesterol (m. p. 79°–80°) $[\alpha]_D$ −114, as 3,5-cholestadiene. The low specific rotation of our hydrocarbon was due perhaps to contamination with isomeric cholestadienes of lesser optical activity. However, this product was of sufficient uniformity for the purpose for which it was prepared, namely, to determine its effects when fed to rats and when implanted subcutaneously in rats and mice.

IMPLANTATION OF 3,5-CHOLESTADIENE PELLETS IN RATS

Thirty healthy young adult Wistar rats, 24 males and 6 females, average weight about 200 gm., were chosen for implantation of 3, 5-cholestadiene pellets. The hydrocarbon was melted, drawn into lightly oiled capillary tubes, allowed to solidify, and extruded. Oil was removed from the cylindrical pellets by gently washing with a few drops of a mixture of equal parts of alcohol and ether. The pellets, 2 mm. in diameter, were cut into lengths of 4.5 mm., and were found to weigh 14-15 mg. Implantation in the rats was effected with a 12-gage trocar, subcutaneously, near the dorsocephalad extremity of the right inguinal fold. The animals were fed Purina dog chow and water and were examined regularly at 2-week intervals. Presence of intact pellets was established by palpation.

Ten rats died or were killed because of illness during the following 12 months. The other 20 rats were killed from 13 to 16 months after pellet implantation. Autopsy of each animal revealed an intact pellet; occasionally a very slight erosion of the sharper edge of the pellet was noted. In no instance was there evidence of hyperplasia. A few pellets were walled off by a thin capsule. Frequently a small area of the subcutaneous fat in the area surrounding the pellet had turned black.

IMPLANTATION OF 3,5-CHOLESTADIENE PELLETS IN MICE

Forty healthy young adult strain C3H male mice, average weight 23 gm., were used for implantation of 3, 5-cholestadiene pellets. These pellets were prepared as described for rats, but were cut into slightly shorter lengths, the average weight between 11 and 12 mg. They were placed in the right inguinal region with the aid of a 12-gage trocar introduced caudally in such a manner as to leave the pellet lying in the areolar tissue cephalad to the inguinal

lymph node. The mice were fed Purina dog chow and water.

Eleven mice died or were killed because of illness during the following 12 months. The other 29 mice were killed and examined 13 to 15 months after implantation of the pellets. The findings were essentially the same as those recorded for pellet implantation in rats, namely, intact pellet showing very little erosion, occasionally a thin encapsulation, and absence of hyperplasia.

IMPLANTATION OF DICHOLESTERYL ETHER PELLETS IN MICE

The least soluble of the reaction products obtained by the dehydration of cholesterol was dicholesteryl ether, the crude isolation of which is described herein. This material was only very slightly soluble in most organic solvents. Chloroform was found to be suitable for recrystallization, from which the ether was obtained as long, silky, white needles, m. p. 204°–205° C., $[\alpha]_D^{21}$–47.0 (in chloroform). The material did not make up into pellets satisfactorily; however, hard lumps were obtained by compression. Pellets weighing 8 to 12 mg. were carved from these lumps with a knife. Forty-four strain C3H male mice 4 months of age were used for pellet implantation. Because of the irregularity of shape of the pellets, they were surgically placed under the skin in the inguinal region with ether anesthesia. The mice were fed Purina dog chow and water and were examined at regular intervals.

Twenty-four animals were autopsied at intervals during the following 12 months; after 12 months the remaining 20 mice were killed and examined. The findings in this series of 44 mice were essentially the same as in the series in which 3,5-cholestadiene pellets were implanted. No evidence of hyperplasia was observed.

FEEDING OF 3,5-CHOLESTADIENE TO RATS

Our experimental diets were designed to contain up to 50 percent of lard; in this respect they approximate the dietary conditions used by Roffo (*2*). The 3,5-cholestadiene was fed to 3 groups of young adult Wistar male rats (10 animals per group) at levels of 1, 10, and 100 times the quantity of this hydrocarbon that could theoretically be formed from the cholesterol and its esters in a 50-percent lard diet. The hydrocarbon was dissolved in warm lard in proportions that provided diets which contained 0.065 percent (group 1), 0.65 percent (group 2), and 6.5 percent (group 3), respectively, of 3,5-cholestadiene, which replaced an equal quantity of lard. A control group (20 rats) was fed the same basic diet, which contained 50 percent of lard without added hydrocarbon. The remaining half of the diet for all groups consisted of the following:

	Percent
Skim-milk powder	24.00
Whole wheat flour	22.35
Brewers' yeast	2.00
Corn oil fortified [1] with vitamins A [2] and D [3]	1.00
Sodium chloride	.50
Ferric citrate	.15

[1] Equivalent in vitamins A and D to 2 percent U. S. P. XI cod-liver oil.
[2] Vitamin A ester was supplied by Distillation Products, Inc., Rochester, N. Y.
[3] Calciferol was supplied by Mead Johnson Co., Evansville, Ind.

The animals were individually housed and fed. Animal weights and food consumption, the latter corrected for spillage, were recorded weekly.

RESULTS

The rats fed the diet containing the lowest level of 3,5-cholestadiene (0.065 percent) experienced the same increase in body weight during the course of this experiment as did the control animals fed a 50-percent lard diet (fig. 1, *A*). The group of rats that received the diet containing the intermediate level of 3,5-cholestadiene (0.65 percent) maintained a constant weight for 6 months and declined slightly thereafter. The group fed the diet containing the highest level of the hydrocarbon (6.5 percent) lost weight regularly except for a temporary recovery trend during the third and fourth months. The mortality rate was much higher in

this last-mentioned group after the sixth month; all the animals died before the end of the eighth month.

The data on the food consumption of the three experimental groups of rats and of the controls are summarized in figure 1, *B*. Here, again, the group fed the diet containing the lowest level of 3,5-cholestadiene closely approximated the control group. The group fed the intermediate concentration of the hydrocarbon consistently consumed less

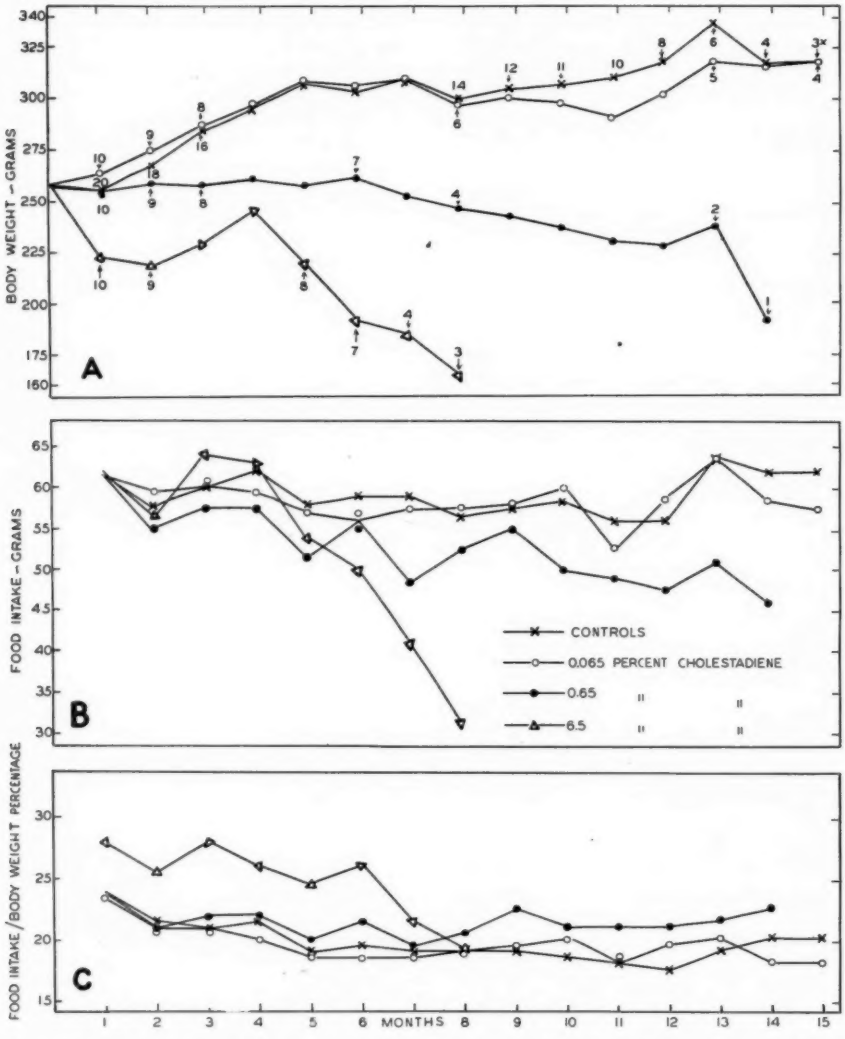

FIGURE 1.—*A*, Average weight curves of three experimental feeding groups and controls; *B*, Average food intake in grams per week; *C*, Average ratio, food intake per week/body weight. Numbers on curves represent surviving animals.

food than either the control group or the low-level-hydrocarbon group; this lowered food consumption was reflected in a correspondingly lower average weight. The group fed the highest level of the hydrocarbon ate more food than any other group during the third and fourth months but thereafter ate less and less as the average weight of the animals decreased.

The ratio of food consumption to body weight, averaged for each group, is recorded in figure 1, *C*. The group fed the highest concentration of the hydrocarbon provided consistently higher ratios of food to body weight. It is noted that this nonconformity with the ratios of the other 3 groups is due to diminished body weight and not to any excessive food consumption. It is apparent that the presence of 6.5 percent of 3,5-cholestadiene in the diet impaired the efficiency of utilization of food as determined by calculation of the ratio of food to body weight. This impairment appears to have been exerted, to a lesser degree, by the diet containing the intermediate level (0.65 percent) of the hydrocarbon.

PATHOLOGY

Gross pathologic findings at the sites of pellet implantation of 3,5-cholestadiene in mice and rats and of dicholesteryl ether in mice have been recorded briefly in the report of each experiment.

In rats fed 3,5-cholestadiene at 3 levels, steatorrhea and diarrhea were common terminal conditions (7 of 30 animals), as well as in the controls (5 of 20 animals). The only other abnormality frequently encountered was bronchiectasis and terminal pneumonia. This condition was found in 11 of 20 control rats and in 16 of 30 animals fed the hydrocarbon. This disease process is common in older animals of this strain, and in the present experiment cannot be attributed to the conditions imposed. Two control animals appeared to have died of otitis media with middle ear extension. All other animals were killed when their condition became poor.

The stomachs and livers of the 50 rats autopsied in this experiment were routinely fixed in Zenker-formol solution. After routine paraffin embedding, they were sectioned and stained with hematoxylin and eosin. Pieces of liver were fixed in dilute formaldehyde (U. S. P. 1:10) and a fat stain, sudan IV, was applied. Some liver blocks were fixed in absolute ethyl alcohol and stained by Best's carmine method for glycogen. Certain organs, in addition to those above-named, from rats which survived for the longest periods, such as heart, lungs, lower gastrointestinal tract, and bone marrow, were sectioned. These specimens were characteristically those of old animals.

Microscopic examination of the structure of the gastric wall from control animals showed all of them to be within the normal range, except three. Two of these showed rather marked submucosal fibrosis; the third showed a moderate hyperkeratosis of the nonglandular stomach at the limiting ridge. Sections of livers from control animals showed variations ranging from normal, nonfatty livers to others which were heavily infiltrated. This infiltration appeared indiscriminately throughout the liver lobule. It was characterized by single or multiple fat droplets which displaced the cell nucleus to one side. No fibrosis was observed. Attempts to correlate the severity of infiltration with the length of time the rats were fed the high fat diet were unsuccessful.

In animals fed 3,5-cholestadiene, the fatty infiltration of the liver resembled in all respects that seen in the control rats. There was no distinction between low, intermediate, or high levels of the hydrocarbon, nor was there any time correlation.

The gastric mucosa from each of 10 rats fed diets of low cholestadiene was examined. Two showed hyperkeratosis of the squamous portion, similar to that seen in two control animals; in a third the process was somewhat more extensive, and a papilloma had developed. The glandular stomach of a fourth animal showed an acute ulcer, and a fifth

showed a few scattered cystic glands. None of these changes can be regarded as significant. Sections of the stomach from 10 rats fed the intermediate level of the hydrocarbon were examined, and 3 showed hyperkeratosis; 1 of these exhibited a papilloma. Stomach sections from 10 rats fed a high cholestadiene diet were examined, and 5 showed hyperkeratotic lesions of the nonglandular stomach near the limiting ridge. Of these, 3 also presented papillomas. The glandular mucosa from 1 animal showed a superficial ulcer.

DISCUSSION

Roffo (*1, 2*) appears to have used upwards of 2,000 rats in feeding experiments employing irradiated or heated cholesterol or heated natural fats. The incidence of advanced gastric lesions in Roffo's numerous experimental groups is not stated. He apparently found no similar gastric changes in many thousands of control rats, but it is not clear at what age the controls were autopsied. Neither is it clear whether the basic ration, food consumption, and body weights of his control rats were comparable with those of the experimental animals. He claimed that irradiating or heating of cholesterol per se, or heating of natural fats caused a degradation of cholesterol to a carcinogenic "oxycholesterol." The chemical changes postulated by Roffo have been reviewed by Cook and Kennaway (*12*) and by Bergmann (*13*) who cast some doubt on his conclusions. Roffo showed many photographs of gross lesions of the forestomach and photomicrographs which were the basis of his diagnosis of carcinomatous ulcers and adenocarcinoma. No evidence was given of metastasis of any gastric lesion, of invasion of neighboring structures, or of transplantability of any "adenocarcinoma." As stated by Klein and Palmer (*14*)—

"on the basis of the changes shown in the photomicrographs, the question may be seriously raised as to whether these lesions were really malignant."

Waterman (*3*) found tumors of the forestomach in 4 of 46 mice fed cholesteryl oleate for about a year. He regarded these lesions as squamous-cell carcinomas, a diagnosis which is not adequately supported by the evidence. The report by Veldstra (*4*) states that Waterman fed 3,5-cholestadiene to an unspecified number of mice, and after 1 year found 4 papillomas of the stomach, 1 of which showed infiltrating properties. No further description of these lesions is available.

In contrast with the findings of Roffo and of Waterman, similar experiments by other investigators have led to essentially negative results. Morris, Larsen, and Lippincott (*8*) fed heated lard, together with an otherwise adequate diet, to rats and found no gastric or hepatic neoplasms. Beck and Peacock (*15*) fed heated fats to rats and found ulceration and papillomatosis of the forestomach, which were attributed to avitaminosis-A. There was no evidence of malignancy in these lesions. From the same laboratory, Kirby (*5*) reported that feeding of heated cholesterol, 20 mg. per day, for 2 years, or 3,5-cholestadiene (quantity not specified) for 18 months,[1] to rats elicited no carcinomatous lesions in either part of the stomach. Whereas this hydrocarbon feeding experiment of Kirby was continued for 18 months, our experiments were self-terminated at 15 months or less. However, the animals fed our graded levels of 3,5-cholestadiene consumed 5.5, 50, and 530 mg. per day per rat. The quantities of the hydrocarbon consumed by rats fed these intermediate and high levels are approximately 2 and 20 times the amount of material fed by Kirby, if it is assumed that the latter fed quantities approximating the 20-mg. doses of heated cholesterol. In spite of much greater quantities of 3,5-cholestadiene fed to our rats, changes in the forestomachs did not differ significantly from those in the controls.

[1] As this paper goes to press, a communication from Kirby (*16*) has appeared in which it is reported that rats fed 25 mg. of 3,5-cholestadiene daily for 2 years, plus an adequate basal diet, developed no gastric tumors.

The wide range of experimental conditions which lead to ulceration, keratosis, and papillomatosis of the forestomach of the rat has been listed by Sugiura (*17*). Inanition, unbalanced diets, deficiencies of vitamins and other dietary essentials, parasites, mechanical irritation, old age, etc., are factors in the development of papillomatosis and ulceration in the forestomach of the rat. It may well be that one or more of these factors escaped recognition in the experiments of Roffo and of Waterman. The rats used in the investigations which gave the negative results cited (*5, 7, 8, 15*), as well as those described in this paper, were maintained on diets which were nutritionally equal or superior to those described by Roffo. Perhaps as Kirby states—

> deliberately unbalanced diets may be needed before a variety of potentially pathogenic agents can cause significant lesions of the stomach.

The absence of definitive criteria of malignancy in the descriptions of gastric lesions by Roffo and by Waterman, the relative ease of induction of keratosis and papillomatosis of the forestomach in rats by a variety of experimental conditions, and the inability of other investigators to induce gastric neoplasia with similar procedures, elicit doubt as to the alleged carcinogenicity of heated cholesterol or heated fats administered orally.

SUMMARY

Three groups of rats were fed diets containing 3,5-cholestadiene at graded levels. No characteristic pathology of gastric or hepatic tissues, which would distinguish one experimental group from another or from the controls, was noted. Hyperkeratosis of the forestomach was rather evenly distributed among all the groups. No papillomas were found in the stomachs of the control rats, whereas 1, 1, and 3 were found in the forestomachs of rats fed the low, intermediate, and high concentrations of the hydrocarbon, respectively.

The highest level of ingested hydrocarbon appeared to exert a low-grade systemic toxicity characterized by loss in weight of the rats; all animals in this group were dead after 8 months. The animals maintained on the diet containing the intermediate level of 3,5-cholestadiene exhibited a similar reaction, but to a much lesser degree.

Pellets of 3,5-cholestadiene, when implanted subcutaneously in Wistar rats and in strain C3H mice, were inert. Autopsies up to 16 months after implantation revealed intact pellets and no evidence of hyperplasia in adjacent tissues.

Dicholesteryl ether, implanted subcutaneously in strain C3H mice in the form of pellets, remained intact and caused no tissue reaction.

3,5-Cholestadiene, administered orally or subcutaneously, presented no evidence of carcinogenicity in these experiments.

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TEST OF DESOXYCHOLIC ACID FOR CARCINOGENICITY IN RATS AND MICE

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INTRODUCTION

During the last decade, many contributors to the cancer literature have suggested that there may be some relationship between the genesis of cancer and the bile acids, hormones, or sterols. The most potent of known carcinogenic hydrocarbons, 20-methylcholanthrene, has been prepared from two of the more predominant bile acids by admittedly drastic laboratory methods. The reactions involved in the laboratory conversion of desoxycholic (3,12-dihydroxycholanic) acid and cholic (3,7,12-trihydroxycholanic) acid to methylcholanthrene consist of four types: oxidation, hydrogenation, cyclization, and dehydrogenation. Similar types of reaction are known to take place in the animal body. Absence of proof of like or similar reactions involved in the metabolism of bile acids or other steroids does not necessarily rule out the possible elaboration of as yet undetectable traces of carcinogenic substances during the normal or perhaps abnormal metabolism of steroids.

Methylcholanthrene was first prepared not by synthesis but by degradation of desoxycholic acid by Wieland and Dane (*1*). The relationship of this hydrocarbon to benzanthracene and its derivatives was recognized, and Cook and Haslewood (*2*) first reported the strong carcinogenicity of methylcholanthrene. Subsequently, Fieser and Newman (*3*) prepared methylcholanthrene by degradation of cholic acid. The discovery that a very potent carcinogenic hydrocarbon could be prepared from bile acids raised the question in several laboratories of the possible carcinogenicity of the bile acids, of which desoxycholic acid received the most attention. Ghiron (*4*) reported that desoxycholic acid, when injected subcutaneously into mice and rats, induced transplantable fibrosarcomas in a high proportion of the animals, but no details are available. Law (*5*) reported a significant increase in the incidence of pulmonary tumors in strain A mice following subcutaneous injection of sodium desoxycholate in olive oil. Mice similarly treated with cholic acid did not differ from the controls. Cook, Kennaway, and Kennaway (*6*) observed sarcomas in three of five surviving mice following repeated subcutaneous injections of desoxycholic acid in sesame oil. These sarcomas failed to grow when transplanted into mice of a mixed stock. On the other hand, Shear (*7*) was unable to induce tumors in mice with this agent or with cholic acid. This lack of agreement may have been the result of differences in technique, strain of test animal, vehicle, etc.

The presence of bile in the small intestine has long been recognized as necessary for optimum absorption of a variety of dietary components. The effects of disruption of normal bile secretion, as a result of disease or of surgical procedures, have been alleviated by the oral administration of bile preparations. The biochemical literature contains many references (*8-15*) demonstrating the efficacy of bile preparations, particularly desoxycholic acid, in facilitating the absorption of lipids, cholesterol, vitamins, hormones, and other substances in cases of disturbed bile secretion, and in the enhancement of the absorption of these substances in the normal animal. These reports describe experiments in which bile preparations or desoxycholic acid have been fed to mice, rats, rabbits, and guinea pigs for relatively short periods, usually less than 100 days. In no instance was there any description of the histologic appearance of the stomachs of the experimental animals.

It became necessary, during the course of relevant experiments in this

[1] Now Major, M. C., A. U. S.

laboratory (*16*, *17*), to investigate the effects of prolonged feeding of desoxycholic acid upon the histologic appearance of the gastric mucosa of rats. Concurrently, the effects of subcutaneously implanted desoxycholic acid pellets in rats and mice were observed. This paper presents the results of these investigations.

EXPERIMENTAL PROCEDURE

Desoxycholic acid, "purified", obtained from a biologic supply house, was subjected to intensive purification as recommended by Sobotka (*18*, *pp. 77–78*). The final product, free of all solvent, melted at 174° C. This pure acid was ground in a mortar, passed through a 40-mesh screen, and preserved in a moisture-free atmosphere. One cannot place too much stress upon the importance of subjecting commercially available "purified" desoxycholic acid to further purification. In several instances we found that samples sold by the trade as pure acid contained as much as 30 percent of impurities.

ORAL ADMINISTRATION

Fifty-six male rats, 6 months of age, were procured from a dealer, who certified them as being of the Wistar strain. These animals varied in weight between 245 and 363 gm. They were divided into 4 groups of 14 rats each, in a manner that resulted in equal total weights for each group. Group 1, which served as a control, received the basic diet which consisted of the following:

	Percent
Skim-milk powder	22.75
Whole-wheat flour	61.52
Brewers' yeast (Harris' medicinal)	4.00
Sodium chloride	1.40
Ferric citrate	.13
Cod-liver oil	2.00
Corn oil	8.20

Groups 2, 3, and 4 were also fed the foregoing diet to which was added 5 mg., 25 mg., or 125 mg. desoxycholic acid per 100 gm. of diet, respectively. The rats were housed individually in screen-bottom cages and were provided with food cups that were practically spill-proof. Individual animal weights and food consumption were recorded at weekly intervals. Average animal weights and food consumption, at monthly intervals, are recorded in figure 1.

SUBCUTANEOUS IMPLANTATION

Desoxycholic acid pellets were cut from the hard lumps that result from the final step of the purification of the acid. These rough pellets were surgically implanted, under ether anesthesia, in the right inguinal region of 25 male and 25 female rats, 10 to 12 weeks of age. The incision was made in the groin, and the pellet was placed anteriorly so that it lay about 3 cm. from the incision. The skin was closed with 1 silk stitch. The implanted pellets ranged from 14 to 19 mg. in weight, with an average of 16.5 mg. Almost invariably a marked induration about the site of the pellet was noted during the first few days. The overlying skin remained intact but appeared reddened in many cases. After 2 weeks the pellet had been completely absorbed, the reaction appeared to have subsided, and healing seemed to be complete in most cases. The animals were examined at regular intervals for signs of any delayed reaction. Nine rats died or were killed because of illness during the 12 months following pellet implantation. Gross examination of the pellet site gave uniformly negative results. The remaining 41 rats were killed and examined 13 to 16 months after insertion of the pellet. Grossly and microscopically, the site of injection was consistently without neoplastic change.

Rough pellets of desoxycholic acid, 11 to 13 mg. in weight, were similarly placed under the skin of 46 strain C3H male mice, 8 weeks of age. Within 48 hours an inflamed area of induration developed about the site of the pellet. This reaction was followed by necrosis, sequestration, and sloughing. Five days after implantation the pellet had been sloughed in 38 cases. Sequestration and sloughing failed to develop in

the other 8 mice; however, the pellet appeared to be completely absorbed, in every case, within 7 days. After 4 months, autopsy of 26 mice that died of a pneumonia epidemic revealed a uniformly normal implantation site. The experiment was terminated at the end of 6 months. The remaining 20 mice showed no evidence of neoplasia.

In order to test further the effects of desoxycholic acid on subcutaneous tissue, it became apparent that the pellet required modification in a manner that would reduce its caustic action and likewise retard its rate of absorption. Accordingly, pellets were prepared which contained equal parts by weight of desoxycholic acid and cholesterol. Equal quantities of cholesterol and the acid were powdered, mixed intimately, and melted. The molten mixture was drawn into lightly oiled capillary tubes, cooled, and extruded. The pellets were rinsed with a mixture of equal parts of alcohol and ether, dried, and cut into suitable lengths for implantation.

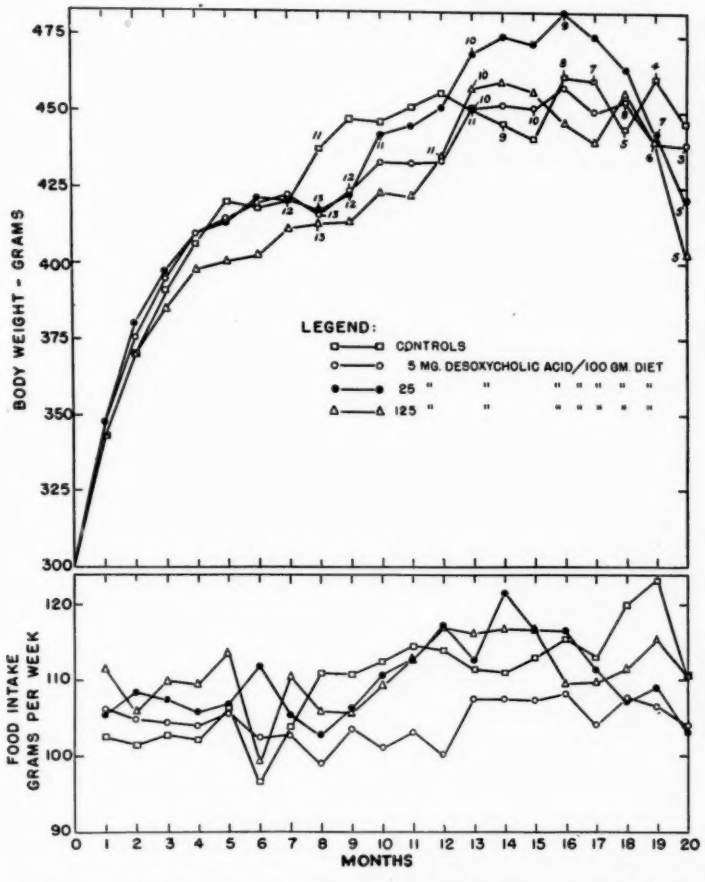

FIGURE 1.—Food intake and body weights of rats on basic diet and on basic diet plus desoxycholic acid.

Fifty-two male Wistar rats 4 months of age received implantations of the modified pellet which was placed, by means of a trocar, in the right groin just cephalad and dorsal to the inguinal fat pad. The pellets weighed 15 to 16 mg. The reaction about the site of the pellet was similar to that noted previously for pellets of desoxycholic acid alone. Seven days later the reaction had subsided, and a movable nodule, roughly 10 mm. in diameter, was noted in each animal. At the end of 2 weeks the nodule was approximately 6 mm. in diameter, on the average. Five weeks after implantation the nodules had shrunk to barely palpable size. From 9 to 12 months after pellet implantation, 12 rats were autopsied. In 6 animals the pellet had completely disappeared; a barely perceptible pellet was found in each of the other 6 rats. The pellet site appeared normal in all animals. The remaining 40 rats, autopsied from 12 to 20 months after pellet implantation, showed no residual pellet, and the areas of implantation were apparently normal.

Similarly, desoxycholic acid-cholesterol pellets were implanted subcutaneously in 50 strain C3H male mice 12 weeks of age. The pellets, which weighed 11 to 13 mg., were placed, with the aid of a trocar, in the right groin just cephalad to the inguinal fat pad and directly under the skin. The reaction about the site of implantation was similar to that encountered when pellets of the pure acid were used. Inflammation, induration, necrosis, sequestration, and sloughing were noted in 21 mice after 10 days. Of the other 29 mice, in which the reaction subsided short of sequestration and sloughing, 16 were found to have soft nodules under the skin after 20 days; absorption of the pellet material appeared to have been effected in 13 mice. Absorption of the pellet in all animals appeared to be complete within 35 days. The experiment was terminated at the end of 8 months. No evidence of hyperplasia at the pellet site was found in any case.

DISCUSSION

On examination of several reports in the literature (*8–14*), relative to oral administration of desoxycholic acid and bile salts to small experimental animals, it would appear that desoxycholic acid is not toxic when given orally. In but one case, insofar as we are aware, was there an exception. Wells, Burrill, and Greene (*15*) found that 0.6 mg. desoxycholic acid per day, when given orally along with testosterone propionate, caused 150-gm. rats to lose weight over a 10-day period. This loss in weight was apparently avoided when the acid was reduced to 0.5 mg. per day. It must be emphasized that, according to our findings, commercially available "purified" desoxycholic acid may contain as much as 30 percent of impurities. In contrast with the findings of Wells, et al., we have found that young adult Wistar rats suffer no ill effects while ingesting as much as 20 mg. of the acid per day (group 4), purified in this laboratory, during 20 months. As is shown in figure 1, the growth curves for the control group and for each of the experimental groups are similar, except that the curve for group 4, which received 125 mg. of the acid per 100 gm. of the diet, is slightly lower than the others from the fourth to the eleventh month. Similarly, the food consumption for all groups was comparable, except that group 2, which was fed the low level of the acid (5 mg./100 gm. diet), appeared to consistently eat slightly less food than did the other groups from the seventh to the eighteenth month. We are of the opinion that these small differences in average weights and food consumption are without significance owing to the relatively small number of rats in each group.

PATHOLOGY

All 56 rats of the control and desoxycholic acid-fed groups were autopsied. Six small papillomas of the forestomach and five superficial ulcers of the glandular stomach, found rather evenly distributed among the several groups,

were considered not to be a result of the experimental procedures. Fourteen animals were found to be normal on gross examination. Many animals had minor lesions, such as superficial ulcers of the foot pad, alopecia, papillomas of the ear on a parasitic basis, and an occasional parasitic cyst in the abdomen. Obesity was common in the control and the acid-fed groups. Nineteen cases of bronchiectasis, usually associated with chronic bronchitis and peribronchial inflammation, were observed. Numerous mucinous cysts of the bronchi, bronchopneumonia, with or without abscesses, and extensive metaplasia of the bronchial epithelium commonly accompanied the bronchiestasis. These lesions of the respiratory tract were rather evenly distributed among the control and acid-fed groups. Eight cases of pyelonephritis were seen, 2 in each group of 14 rats. Three cases of otitis media interna, randomly distributed among the several groups, were encountered. All of these subacute and chronic inflammatory processes were accompanied by hyperplasia of the spleen and bone marrow. A few neoplasms were observed in old rats. One animal in group 2 showed a hygroma. A carcinoma of the testis was found in a rat in group 3. A rat in group 4 had a spindle-cell sarcoma on a foot pad with retroperitoneal metastases. Two anaplastic carcinomas of the lung were noted in animals in groups 2 and 4, respectively. In our opinion, it is doubtful whether these neoplasms were attributable to the imposed experimental conditions.

SUMMARY

Three groups of rats were fed diets containing desoxycholic acid at graded levels for as long as 20 months without evidence of toxic effects. No characteristic pathology, which would distinguish one experimental group from another or from the controls, was noted in the gastric tissues.

Pellets of desoxycholic acid, implanted subcutaneously in rats, incited a temporary local tissue reaction which subsided within 2 weeks. Autopsy of 41 animals after 13 to 16 months revealed neither gross nor microscopic evidence of neoplasia or hyperplasia. Subcutaneous implantation of pellets consisting of equal parts of the acid and cholesterol likewise led to negative results as far as tumor genesis was concerned.

Subcutaneous implantation of desoxycholic acid pellets in 46 strain C3H mice led to necrosis, sequestration, and sloughing of the pellet in most cases. Twenty-six mice which survived for 6 months showed no evidence of hyperplasia at the pellet site. Similar implantation of acid-cholesterol pellets was followed by sloughing in about half the mice. Pellets that were retained in the remainder of the mice appeared to be completely absorbed within 35 days. These mice, autopsied 8 months later, showed no reaction at the pellet site.

Desoxycholic acid, as administered in these experiments, showed no evidence of carcinogenicity.

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